

**JOINT INVENTORS**

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Jill E. Uhl

**APPLICATION FOR  
UNITED STATES LETTERS PATENT**

**S P E C I F I C A T I O N**

**TO ALL WHOM IT MAY CONCERN:**

Be it known that we, John Ford a citizen of United States, residing at 2763 S. Norfolk, #210, in the City of San Matero and State of California, 94403 and Julio J. Mulero a citizen of United States, residing at 892 Southampton Drive, in the City of Palo Alto and State of California, 94303 have invented a new and useful METHODS AND MATERIALS RELATING TO NOVEL CD39-LIKE POLYPEPTIDES, of which the following is a specification.

# METHODS AND MATERIALS RELATING TO NOVEL CD39-LIKE POLYPEPTIDES

## 1. RELATED APPLICATIONS

5 This patent application is a continuation-in-part of U.S. patent application Serial No. 09/273,447 filed March 19, 1999 which is a continuation-in-part of U.S. patent application Serial No. 09/122,449 filed July 24, 1998 and also a continuation-in-part of U.S. patent application Serial No. 09/244,444 [ATTORNEY DOCKET NO. 20411-745CON1] filed February 4, 1999, which in  
10 turn is a continuation-in-part of U.S. patent application Serial No. 09/118,205 (Attorney Docket No. 20411-745), filed July 16, 1998, the disclosures of all of which are incorporated by reference herein in their entirety.

## 2. FIELD OF THE INVENTION

15 This invention relates in general to novel polynucleotides isolated from cDNA libraries of human fetal liver-spleen and macrophages and to polypeptides encoded by these polynucleotides. In particular, the invention relates to a human CD39-like protein with homologies to ATP diphosphohydrolases and variants thereof.

## 3. BACKGROUND

CD39 (cluster of differentiation 39) is a cell-surface molecule recognized by a "cluster" of monoclonal antibodies that can be used to identify the lineage or stage of differentiation of lymphocytes and thus to distinguish one  
25 class of lymphocytes from another. This CD39 molecule was originally defined as a B lymphocyte marker (Rowe, M., et al. Int. J. Cancer 29:373 (1982)). Subsequent studies have shown CD39 to be a marker for a distinct subset of activated lymphocytes within the allosensitized CD8-positive cytotoxic cells (Gouttefangeas C., et al., Eur. J. Immunol. 22:2681 (1992)). Outside of lymphoid  
30 tissue, CD39 can be found in quiescent vascular endothelial cells (Kansas, G. S., et al., J. Immunol. 146:2235 (1991)) and throughout rat brain in the neurons

of the cerebral cortex, hippocampus, and cerebellum, as well as in glial cells (Wang, T-F. and Guidotti, G., Brain Res. 790:318 (1998)).

CD39 is a 510-amino acid protein with a predicted molecular mass of 57 kDa. However, because of heavy glycosylation at asparagine residues (six potential N-glycosylation sites) the molecule displays a mobility closer to 100 kDa (Maliszewski, C. R., et al., J. Immunol. 153:3574 (1994)). CD39 contains two hydrophobic regions, one near the amino terminus and the other near the carboxyl terminus which are believed to be transmembrane regions.

Reports that several ATP Diphosphohydrolases (ATPDases) share amino acid sequence homology with CD39 have been substantiated by showing that CD39 is itself an ATPDase (Wang, T- F., et al., J. Biol. Chem. 271:9898 (1996); Kaczmarek, E., et al., J. Biol. Chem. 271:33116 (1996)). Since CD39 is a plasma membrane-bound enzyme, CD39 has been termed an "ecto-ATPase," but CD39 is more often referred to as an "ecto-apyrase" because of the reduced rate of hydrolysis of ADP when compared with ecto-ATPDases.

This activity has shown to modulate platelet reactivity and aggregation in response to vascular injury. During vascular injury, activated platelets aggregate forming an occlusive thrombus. Excessive platelet accumulation at sites of vascular injury can contribute to vessel occlusion. Endothelial cells respond to the potentially occlusive effects of platelet aggregation by several mechanisms. One of these mechanisms results ecto-apyrase-mediated removal of ADP, which in turn eliminates platelet reactivity and recruitment. It is now known that the endothelial ecto-apyrase responsible for this ADP removal is CD39 (Marcus, A. J., et al., J. Clin. Invest. 99:1351 (1997)).

Recently, CD39 was engineered to produce a soluble form of the molecule. This soluble CD39 was shown to display the same nucleotidase activity as the membrane-bound molecule (Gayle, R. B., et al., J. Clin. Invest. 101:1851 (1998)). Intravenously administered soluble CD39 also remained active in mice for an extensive period of time, indicating that soluble CD39 could

be useful as a inhibitor of platelet aggregation in the prophylaxis or treatment of platelet-mediated thrombotic conditions.

Platelet aggregation inhibitors (antithrombotic agents) decrease the formation or the action of chemical signals that promote platelet aggregation. Currently available antithrombotic agents include aspirin, ticlopidine, and dipyridamole. These agents have proven beneficial in the prevention and treatment of occlusive cardiovascular diseases, including myocardial infarction, cerebral ischemia, angina. Antithrombotic therapy has also been used in the maintenance of vascular grafts.

Myocardial infarction is the development of necrosis of the myocardium (the middle muscular layer of the heart wall) due to a critical imbalance between oxygen and myocardial demand. The most common cause of acute myocardium infarction is narrowing of the epicardial blood vessels due to atheromatous plaques. Plaque rupture with subsequent exposure of basement membrane results in platelet aggregation and thrombus formation, which can result in partial or complete occlusion of the vessel and subsequent myocardial ischemia.

In cerebral ischemia, inadequate blood flow results from an occlusion in a blood vessel or hemorrhaging. In the latter case, excessive bleeding in one area of the brain deprives another area of blood. If the damage occurs in a singular small area, "transient" or "focused" cerebral ischemia results. When a major artery is blocked (carotid artery) global or diffused ischemia results. The primary medical strategy for secondary prevention of stroke is antiplatelet therapy. Aspirin is currently employed for reducing the risk of recurrent transient ischemic attacks or stroke in men who have transient ischemia of the brain due to fibrin emboli.

Each year, thousands of patients suffer a decline in blood flow to one or more limbs. Without sufficient blood flow, and, unless blood flow can be restored in time, the limb must be amputated. In some cases, grafts from the patient's veins can be used to form new arteries. However, in cases where the

quality of the veins is poor, polymeric vascular grafts are typically used. The polymeric grafts are inherently thrombogenic as the blood constituents passing through the grafts become activated and tend to form clots. Efforts to line the grafts with endothelial cells can reduce blood clotting, but better results are obtained when antithrombotic therapy is employed.

Angina pectoris is a characteristic chest pain caused by inadequate blood flow through the blood vessels of the myocardium. The imbalance between oxygen delivery and utilization may result from a spasm of the vascular smooth muscle or from obstruction of blood vessels caused by atherosclerotic lesions. Three classes of drugs have been shown to be effective in treating angina: nitrates, beta-blockers and calcium channel blockers. Currently, the antithrombotics dipyridamole and aspirin are employed to prophylactically treat angina pectoris.

Ecto-apyrases, such as CD39, offer a number of advantages over several of the standard antithrombotics. For example, aspirin treatment controls the prothrombotic action of thromboxane; however, aspirin also prevents formation of antithrombotic prostacyclin, which limits aspirin's efficacy. Another antithrombotic, endothelium-derived relaxing factor (nitric oxide; "EDRF/NO"), is inhibited in vitro and in vivo by hemoglobin after its rapid diffusion into erythrocytes. In contrast, CD39 is aspirin-insensitive and completely inhibits platelet reactivity even when eicosanoid and EDRF/NO production are blocked.

CD39's ATPase activity also implicates CD39 in the modulation of neurotransmission. ATP is a major purinergic neurotransmitter that is often co-released into the synaptic cleft with several neurotransmitters. Responses to ATP are mediated by specific plasma membrane receptors, called P2 purinergic receptors (Dubyak, G. R. and El-Motassim, C. Am J. Physiol. 34:C577-C606 (1993)). The distribution of CD39 in the rat brain indicates that CD39 plays a role in terminating P2 purinergic neurotransmission (Wang, T. F. and Guidotti, G., Brain Res. 790:318 (1998)). Furthermore, a decrease in ecto-apyrase activity is believed to lead to an accumulation of the excitatory

neurotransmitter, extracellular ATP, as well as a deficiency of the endogenous anticonvulsant extracellular adenosine.

The chromosomal localization of CD39 provides additional support for a role in modulation of neurotransmission. More specifically, CD39 has been mapped to chromosome 10q 23.1-24.1 (Maliszewski, C. R., et al., J. Immunol. 153:3574 (1994)), and this site overlaps with the susceptibility locus for human partial epilepsy with audigenic symptoms (Ottman, R. et al., Nature Genet. 10:56 (1995)). This co-localization of the CD39 gene and the susceptibility locus has led to the hypothesis that decrease in ecto-apyrase activity in the brain is the primary cause of partial epilepsy (Wang T-F., et al., Mol. Brain Res. 47:295 (1997)).

A screen for human cDNAs that hybridize to cosmids from the human chromosome 9q34 region lead to the identification of a transcript with high homology to a chicken muscle ecto-ATPase (60% identity) and the ecto-apyrase CD39 (41% amino acid identity) (Chadwick, B. P., Mamm. Genome 8:668 (1997)). This gene, designated "CD39-like-1 gene" (CD39L1), has a higher degree of homology to CD39 than does chicken muscle ecto-ATPase. The biological activity of this protein has not been tested but on the basis of the high amino acid homology, CD39L1 is believed to be a new member of the ecto-ATPase family. Recently, a mouse gene with homology to NTPases was cloned and sequenced (Acc. No. AF006482) by Chadwick et al. (Mamm. Gen. 9:162-164 (1998).)

#### 4. SUMMARY OF THE INVENTION

The invention is based on polynucleotides isolated from cDNA libraries prepared from human fetal liver-spleen and macrophages. The compositions of the present invention include novel isolated polypeptides with apyrase and/or NDPase activity, in particular, novel human CD39-like polypeptides, and active variants thereof, isolated polynucleotides encoding such polypeptides, including recombinant DNA molecules, cloned genes or

degenerative variants thereof, especially naturally occurring variants such as allelic variants, antisense polynucleotide molecules, and antibodies that specifically recognize one or more epitopes present on such polypeptides, as well as hybridomas producing such antibodies.

5           The compositions of the invention additionally include vectors, including expression vectors, containing the polynucleotides of the invention, cells genetically engineered to contain such polynucleotides and cells genetically engineered to express such polynucleotides.

10           The polynucleotides of the invention include naturally occurring or wholly or partially synthetic DNA, e.g., cDNA and genomic DNA, and RNA, e.g., mRNA. The isolated polynucleotides of the invention include a polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO. 3. The isolated polynucleotides of the invention further include a polynucleotide comprising the nucleotide sequence of SEQ ID NO. 2. The polynucleotides of  
15           the invention also include polynucleotides that encode polypeptides with a biological activity of the polypeptide of SEQ ID NO. 3 (including apyrase or NTPase activity) and that hybridize under stringent hybridization conditions to the complement of (a) the nucleotide sequence of SEQ ID NO. 2, or (b) a nucleotide sequence encoding the amino acid sequence of SEQ ID NO. 3; a  
20           polynucleotide which is an allelic variant of any polynucleotide recited above; a polypeptide which has at least 80% sequence identity to a polynucleotide of SEQ ID NO. 2; or a polynucleotide that encodes a polypeptide comprising at least one CD39-like domain, e.g. catalytic domain.

25           The polynucleotides of the invention additionally include the complement of any of the polynucleotides recited above.

30           One polynucleotide according to the invention encodes a novel CD39-like protein having the amino acid sequence shown in Figure 2 (SEQ ID NO. 3), which has been designated CD39-L66 and is an isoform of the CD39-L4. The invention also provides a polynucleotide including a nucleotide sequence that is substantially equivalent to this polynucleotide. Polynucleotides according

to the invention can have at least about 80%, more typically at least about 90%, and even more typically at least about 95%, sequence identity to a polynucleotide encoding a polypeptide including SEQ ID NO. 3.

A further aspect of the invention is the development of novel CD39-L4 variants which have improved ADPase activity compared to wild type CD39-L4 (SEQ ID NO: 5). A preferred variant, designated ACRIII herein, has the amino acid sequence set forth in SEQ ID NO: 7. The invention further provides polypeptides comprising at least one amino acid substitution selected from the group consisting of: D168-T, S170-Q and L175-F, wherein said substitution(s) result in increased ADPase activity of the polypeptide. One preferred embodiment is the polypeptide having the sequence set forth in SEQ ID NO: 7, which is a variant CD39-L4 containing all three substitutions that has been designated ACRIII. Alternatively, instead of making the specific D168-T, S170-Q and/or L175-F substitution(s), substitution of amino acids with similar properties is contemplated. Additional conservative substitutions at amino acid positions other than D168, S170 and/or L175 are further contemplated. For example, all of the corresponding amino acids from CD39 could be substituted for amino acids 167-181 of CD39-L66 or CD39-L4.

Polynucleotides encoding these variants, vectors and host cells comprising such polynucleotides, methods of using such host cells to produce polypeptides, and other therapeutic products comprising the polypeptides (including fusion proteins in which the ACRIII is fused to a heterologous peptide or polypeptide, such as an immunoglobulin constant region, or derivatives in which ACRIII is modified by water soluble polymers to increase its half-life) are also comprehended by the invention, as are methods of treating a subject suffering from a disorder relating to thrombosis, coagulation or platelet aggregation by administering such therapeutic products.

Gene therapy techniques are also provided to modulate disease states associated with CD39-L4 expression and/or biological activity. Delivery of a functional CD39-L4 gene to appropriate cells is effected ex vivo, in situ, or



in vivo by use of vectors, and more particularly viral vectors (e.g., adenovirus, adeno-associated virus, or a retrovirus), or ex vivo by use of physical DNA transfer methods (e.g., liposomes or chemical treatments).

The invention also relates to methods for producing polypeptides of the invention comprising growing a culture of cells of the invention in a suitable culture medium under conditions permitting expression of the desired polypeptide, and purifying the protein from the cells or the culture medium. Preferred embodiments include those in which the protein produced by such process is a mature form of the protein.

Protein compositions of the present invention, including therapeutic compositions, comprise polypeptides of the invention and optionally an acceptable carrier, such as a hydrophilic (e.g., pharmaceutically acceptable) carrier.

Polynucleotides according to the invention have numerous applications in a variety of techniques known to those skilled in the art of molecular biology. These techniques include use as hybridization probes, use as oligomers for PCR, use for chromosome and gene mapping, use in the recombinant production of protein, and use in generation of anti-sense DNA or RNA, their chemical analogs and the like. For example, because the expression of CD39-like mRNA is largely restricted to macrophages, polynucleotides of the invention can be used as hybridization probes to detect the presence of macrophage mRNA in a sample using, e.g., in situ hybridization.

In other exemplary embodiments, the polynucleotides are used in diagnostics as expressed sequence tags for identifying expressed genes or, as well known in the art and exemplified by Vollrath et al., Science 258:52-59 (1992), as expressed sequence tags for physical mapping of the human genome.

A polynucleotide according to the invention can be joined to any of a variety of other nucleotide sequences by well-established recombinant DNA techniques (see Sambrook J et al. (1989) Molecular Cloning: A Laboratory

Manual, Cold Spring Harbor Laboratory, NY). Useful nucleotide sequences for joining to polypeptides include an assortment of vectors, e.g., plasmids, cosmids, lambda phage derivatives, phagemids, and the like, that are well known in the art. Accordingly, the invention also provides a vector including a polynucleotide of the invention and a host cell containing the polynucleotide. In general, the vector contains an origin of replication functional in at least one organism, convenient restriction endonuclease sites, and a selectable marker for the host cell. Vectors according to the invention include expression vectors, replication vectors, probe generation vectors, and sequencing vectors. A host cell according to the invention can be a prokaryotic or eukaryotic cell and can be a unicellular organism or part of a multicellular organism.

The polypeptides according to the invention can be used in a variety of conventional procedures and methods that are currently applied to other proteins. For example, a polypeptide of the invention can be used to generate an antibody which specifically binds the polypeptide. The polypeptides of the invention having ATPDase activity are also useful for inhibiting platelet aggregation and can therefore be employed in the prophylaxis or treatment of pathological conditions caused by the inflammatory response. The polypeptides of the invention can also be used as molecular weight markers, and as a food supplement.

Another aspect of the invention is an antibody that specifically binds the polypeptide of the invention. Such antibodies can be either monoclonal or polyclonal antibodies, as well fragments thereof and humanized forms or fully human forms, such as those produced in transgenic animals. The invention further provides a hybridoma that produces an antibody according to the invention.

Antibodies of the invention are useful for detection and/or purification of the polypeptides of the invention.

Methods are also provided for preventing, treating or ameliorating a medical condition, including thrombotic diseases, which comprises

administering to a mammalian subject, including but not limited to humans, a therapeutically effective amount of a composition comprising a polypeptide of the invention or a therapeutically effective amount of a composition comprising a binding partner of (e.g., antibody specifically reactive for) CD39-like polypeptides of the invention. The mechanics of the particular condition or pathology will dictate whether the polypeptides of the invention or binding partners (or inhibitors) of these would be beneficial to the individual in need of treatment.

The invention also provides a method of inhibiting platelet function comprising administering a CD39-L4 polypeptide of the invention to a medium comprising platelets. According to this method, polypeptides of the invention can be administered to produce an *in vitro* or *in vivo* inhibition of platelet function. A polypeptide of the invention can be administered *in vivo* as an antithrombotic agent alone or as an adjunct to other therapies.

The invention also provides methods for detecting or quantitating the presence of the polynucleotides or polypeptides of the invention in a tissue or fluid sample, and corresponding kits that comprise suitable polynucleotide probes or antibodies, together with an optional quantitative standard. Such methods and kits can be utilized as part of prognostic and diagnostic evaluation of patients and for the identification of subjects exhibiting a predisposition to platelet mediated conditions.

The invention also provides methods for the identification of compounds that modulate (i.e. increase or decrease) the expression or activity of the polynucleotides and/or polypeptides of the invention. Such methods can be utilized, for example, for the identification of compounds and other substances that interact with (e.g., bind to) the polypeptides of the invention, and assays for identifying compounds and other substances that enhance or inhibit the activity of the polypeptides of the invention, such assays comprising the step of measuring activity of such polypeptides in the presence and absence of the test compound.

## 5. BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows polynucleotide sequences according to the invention. SEQ ID NO:1 was obtained from the b2HFLS20W cDNA library using standard pcr, sequencing by hybridization signature analysis, and single pass gel sequencing technology. A- adenosine; C-cytosine; G-guanosine; T-thymine. Ambiguous positions are designated as follows: R indicates A or G; M indicates A or C; W indicates A or T; Y indicates C or T; S indicates C or G; K indicates G or T; V indicates A or C or G; H indicates A or C or T; D indicates A or G or T; B indicates C or G or T; and N indicates any of the four bases.

SEQ ID NO:2 is an extended version of SEQ ID NO:1 which was obtained as described in Example 34.

FIG. 2 shows an amino acid sequence corresponding to the polynucleotide sequence of SEQ ID NO:2. This sequence is designated as SEQ ID NO:3. The open reading frame encoding SEQ ID NO:3 begins at nucleotide 246 (numbered from the 5' end) of SEQ ID NO:2. A- Alanine; R- Arginine; N- Asparagine; D- Aspartic Acid; C- Cysteine; E- Glutamic Acid; Q- Glutamine; G- Glycine; H- Histidine; I- Isoleucine; L- Leucine; K- Lysine; M- Methionine; F- Phenylalanine; P- Proline; S- Serine; T- Threonine; W- Tryptophan; Y- Tyrosine; V- Valine; X - any of the twenty amino acids.

FIG. 3 shows the amino acid sequence alignment of SEQ ID NO:3 (identified as "246 prot") and human CD39 ("CD39Human.seq"). The amino acid residues are designated as for FIG. 2. The alignment was generated using the J. Hein method with the PAM250 residue weight table. Gaps are indicated by dashes; residues that are identical between the two sequences (within 1 distance unit) are boxed.

FIG. 4 shows the amino acid sequence alignment of SEQ ID NO:3 (identified as "264 prot") and murine NTPase ("mur ntpase"). The amino acid residues are designated as for FIG. 2. The alignment was generated as discussed for FIG. 3. Gaps are indicated by dashes; residues that are identical between the two sequences (within 1 distance unit) are boxed.

FIG. 5 shows the apyrase conserved regions (ACR) in CD39-L4 in bold. ACR I starts at Phe 53, ACR II starts at Pro 124 and ACR III starts at Met 167. The boxed sections highlight the amino acid substitutions that were made in the wild type CD39-L4 amino acid sequence to form mutants designated ACRI, ACRII and ACRIII.

FIG. 6 (SEQ ID NOS: 6 and 7) shows the nucleotide and corresponding amino acid sequences of a preferred ACRIII mutant containing the following substitutions in the wild type CD39-L4 amino acid sequence: D168-T, S170-Q and L175-F.

FIG. 7 shows the ADPase activity of CD39-L4 variants ACRI, ACRII and ACRIII in comparison to wild type CD39-L4: (1) CD39-L4 ACR I mutant; (2) CD39-L4 ACR II mutant; (3) CD39-L4 ACR III mutant; (4) CD39-L4 wild type; (5) sCD39; and (6) pSecTag2 vector (Invitrogen).

## 6. DETAILED DESCRIPTION

### 6.1 Definitions

The term "nucleotide sequence" refers to a heteropolymer of nucleotides or the sequence of these nucleotides. The terms "nucleic acid" and "polynucleotide" are also used interchangeably herein to refer to a heteropolymer of nucleotides. Generally, nucleic acid segments provided by this invention may be assembled from fragments of the genome and short oligonucleotide linkers, or from a series of oligonucleotides, to provide a synthetic nucleic acid which is capable of being expressed in a recombinant transcriptional unit comprising regulatory elements derived from a microbial or viral operon.

An "oligonucleotide fragment" or a "polynucleotide fragment", "portion," or "segment" is a stretch of polypeptide nucleotide residues which is long enough to use in polymerase chain reaction (PCR) or various hybridization

procedures to identify or amplify identical or related parts of mRNA or DNA molecules.

"Oligonucleotides" or "nucleic acid probes" are prepared based on the cDNA sequence provided in the present invention. Oligonucleotides comprise portions of the DNA sequence having at least about 15 nucleotides and usually at least about 20 nucleotides. Nucleic acid probes comprise portions of the sequence having fewer nucleotides than about 6 kb, usually fewer than about 1 kb. After appropriate testing to eliminate false positives, these probes may be used to determine whether mRNAs are present in a cell or tissue or to isolate similar nucleic acid sequences from chromosomal DNA as described by Walsh PS et al (1992 PCR Methods Appl 1:241-250).

The term "probes" includes naturally occurring or recombinant single- or double-stranded nucleic acids or chemically synthesized nucleic acids. They may be labeled by nick translation, Klenow fill-in reaction, PCR or other methods well known in the art. Probes of the present invention, their preparation and/or labeling are elaborated in Sambrook J et al (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, NY; or Ausubel FM et al (1989) Current Protocols in Molecular Biology, John Wiley & Sons, New York NY, both incorporated herein by reference.

The term "stringent" is used to refer to conditions that are commonly understood in the art as stringent. An exemplary set of conditions include a temperature of 60-70 oC, (preferably about 65 oC) and a salt concentration of 0.70 M to 0.80 M (preferably about 0.75M). Further exemplary conditions include, hybridizing conditions that (1) employ low ionic strength and high temperature for washing, for example, 0.015 M NaCl/0.0015 M sodium citrate/0.1% SDS at 50(C.; (2) employ during hybridization a denaturing agent such as formamide, for example, 50% (vol/vol) formamide with 0.1% bovine serum albumin/0.1% Ficoll/0.1% polyvinylpyrrolidone/50 mM sodium phosphate buffer at pH 6.5 with 750 mM NaCl, 75 mM sodium citrate at 42(C; or (3) employ 50% formamide, 5 x SSC (0.75 M NaCl, 0.075 M Sodium pyrophosphate, 5 x

Denhardt's solution, sonicated salmon sperm DNA (50 g/ml), 0.1% SDS, and 10% dextran sulfate at 42°C, with washes at 42°C in 0.2 x SSC and 0.1% SDS.

The term "recombinant," as used herein, means that a polypeptide or protein is derived from recombinant (e.g., microbial or mammalian) expression systems. "Microbial" refers to recombinant polypeptides or proteins made in bacterial or fungal (e.g., yeast) expression systems. As a product, "recombinant microbial" defines a polypeptide or protein essentially free of native endogenous substances and unaccompanied by associated native glycosylation. Polypeptides or proteins expressed in most bacterial cultures, e.g., *E. coli*, will be free of glycosylation modifications; polypeptides or proteins expressed in yeast will have a glycosylation pattern different from that expressed in mammalian cells.

The term "recombinant expression vehicle or vector" refers to a plasmid or phage or virus or vector, for expressing a polypeptide from a DNA (RNA) sequence. The expression vehicle can comprise a transcriptional unit comprising an assembly of (1) a genetic element or elements having a regulatory role in gene expression, for example, promoters or enhancers, (2) a structural or coding sequence which is transcribed into mRNA and translated into protein, and (3) appropriate transcription initiation and termination sequences. Structural units intended for use in yeast or eukaryotic expression systems preferably include a leader sequence enabling extracellular secretion of translated protein by a host cell. Alternatively, where recombinant protein is expressed without a leader or transport sequence, it may include an N-terminal methionine residue. This residue may or may not be subsequently cleaved from the expressed recombinant protein to provide a final product.

"Recombinant expression system" means host cells which have stably integrated a recombinant transcriptional unit into chromosomal DNA or carry the recombinant transcriptional unit extrachromosomally. The cells can be prokaryotic or eukaryotic. Recombinant expression systems as defined herein will express heterologous polypeptides or proteins upon induction of the

regulatory elements linked to the DNA segment or synthetic gene to be expressed.

The term "open reading frame," ORF, means a series of triplets coding for amino acids without any termination codons and is a sequence translatable into protein.

The term "expression modulating fragment," EMF, means a series of nucleotide molecules which modulates the expression of an operably linked ORF or EMF.

As used herein, a sequence is said to "modulate the expression of an operably linked sequence" when the expression of the sequence is altered by the presence of the EMF. EMFs include, but are not limited to, promoters, and promoter modulating sequences (inducible elements). One class of EMFs are fragments which induce the expression of an operably linked ORF in response to a specific regulatory factor or physiological event.

As used herein, an "uptake modulating fragment," UMF, means a series of nucleotide molecules which mediate the uptake of a linked DNA fragment into a cell. UMFs can be readily identified using known UMFs as a target sequence or target motif with the computer-based systems described above.

The presence and activity of a UMF can be confirmed by attaching the suspected UMF to a marker sequence. The resulting nucleic acid molecule is then incubated with an appropriate host under appropriate conditions and the uptake of the marker sequence is determined. As described above, a UMF will increase the frequency of uptake of a linked marker sequence.

"Active" refers to those forms of the polypeptide which retain the biologic and/or immunologic activities of any naturally occurring polypeptide.

"Naturally occurring polypeptide" refers to polypeptides produced by cells that have not been genetically engineered and specifically contemplates various polypeptides arising from post-translational modifications of the



polypeptide including, but not limited to, acetylation, carboxylation, glycosylation, phosphorylation, lipidation and acylation.

"Derivative" refers to polypeptides chemically modified by such techniques as ubiquitination, labeling (e.g., with radionuclides or various enzymes), pegylation (derivatization with polyethylene glycol) and insertion or substitution by chemical synthesis of amino acids such as ornithine, which do not normally occur in human proteins.

"Recombinant variant" refers to any polypeptide differing from naturally occurring polypeptides by amino acid insertions, deletions, and substitutions, created using recombinant DNA techniques. Guidance in determining which amino acid residues may be replaced, added or deleted without abolishing activities of interest, such as cellular trafficking, may be found by comparing the sequence of the particular polypeptide with that of homologous peptides and minimizing the number of amino acid sequence changes made in regions of high homology.

Preferably, amino acid "substitutions" are the result of replacing one amino acid with another amino acid having similar structural and/or chemical properties, such as the replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, or a threonine with a serine, i.e., conservative amino acid replacements. "Insertions" or "deletions" are typically in the range of about 1 to 5 amino acids. The variation allowed may be experimentally determined by systematically making insertions, deletions, or substitutions of amino acids in a polypeptide molecule using recombinant DNA techniques and assaying the resulting recombinant variants for activity.

As used herein, "substantially equivalent" can refer both to nucleotide and amino acid sequences, for example a mutant sequence, that varies from a reference sequence by one or more substitutions, deletions, or additions, the net effect of which does not result in an adverse functional dissimilarity between the reference and subject sequences. Typically, such a mutant sequence varies from one of those listed herein by no more than about

20% (i.e., the number of substitutions, additions, and/or deletions in a mutant sequence, as compared to the corresponding listed sequence, divided by the total number of residues in the mutant sequence is about 0.2 or less). Such a mutant sequence is said to have 80% sequence identity to the listed sequence.

5 In one embodiment, a mutant sequence of the invention varies from a listed sequence by no more than 10% (90% sequence identity), in a variation of this embodiment, by no more than 5% (95% sequence identity), and in a further variation of this embodiment, by no more than 2% (98% sequence identity). Mutant amino acid sequences according to the invention generally have at least  
10 95% sequence identity with a listed amino acid sequence, whereas mutant nucleotide sequence of the invention can have lower percent sequence identities. For the purposes of the present invention, sequences having substantially equivalent biological activity and substantially equivalent expression characteristics are considered substantially equivalent. For the  
15 purposes of determining equivalence, truncation of the mature sequence should be disregarded.

Where desired, an expression vector may be designed to contain a "signal or leader sequence" which will direct the polypeptide through the membrane of a cell. Such a sequence may be naturally present on the  
20 polypeptides of the present invention or provided from heterologous protein sources by recombinant DNA techniques.

A polypeptide "fragment," "portion," or "segment" is a stretch of amino acid residues of at least about 5 amino acids, often at least about 7 amino acids, typically at least about 9 to 13 amino acids, and, in various embodiments,  
25 at least about 17 or more amino acids. To be active, any polypeptide must have sufficient length to display biologic and/or immunologic activity.

Alternatively, recombinant variants encoding these same or similar polypeptides may be synthesized or selected by making use of the "redundancy" in the genetic code. Various codon substitutions, such as the silent changes  
30 which produce various restriction sites, may be introduced to optimize cloning

into a plasmid or viral vector or expression in a particular prokaryotic or eukaryotic system. Mutations in the polypeptide sequence may be reflected in the polypeptide or domains of other peptides added to the polypeptide to modify the properties of any part of the polypeptide, to change characteristics such as ligand-binding affinities, interchain affinities, or degradation/turnover rate.

"Activated" cells as used in this application are those which are engaged in extracellular or intracellular membrane trafficking, including the export of neurosecretory or enzymatic molecules as part of a normal or disease process.

The term "purified" as used herein denotes that the indicated nucleic acid or polypeptide is present in the substantial absence of other biological macromolecules, e.g., polynucleotides, proteins, and the like. In one embodiment, the polynucleotide or polypeptide is purified such that it constitutes at least 95% by weight, more preferably at least 99.8% by weight, of the indicated biological macromolecules present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 1000 daltons, can be present).

The term "isolated" as used herein refers to a nucleic acid or polypeptide separated from at least one other component (e.g., nucleic acid or polypeptide) present with the nucleic acid or polypeptide in its natural source. In one embodiment, the nucleic acid or polypeptide is found in the presence of (if anything) only a solvent, buffer, ion, or other component normally present in a solution of the same. The terms "isolated" and "purified" do not encompass nucleic acids or polypeptides present in their natural source.

The term "infection" refers to the introduction of nucleic acids into a suitable host cell by use of a virus or viral vector.

The term "transformation" means introducing DNA into a suitable host cell so that the DNA is replicable, either as an extrachromosomal element, or by chromosomal integration.

The term "transfection" refers to the taking up of an expression vector by a suitable host cell, whether or not any coding sequences are in fact expressed.

The term "intermediate fragment" means a nucleic acid between 5 and 1000 bases in length, and preferably between 10 and 40 bp in length.

Each of the above terms is meant to encompass all that is described for each, unless the context dictates otherwise.

#### 6.4 Hybridization Conditions

Suitable hybridization conditions may be routinely determined by optimization procedures or pilot studies. Such procedures and studies are routinely conducted by those skilled in the art to establish protocols for use in a laboratory. See e.g., Ausubel et al., *Current Protocols in Molecular Biology*, Vol. 1-2, John Wiley & Sons (1989); Sambrook et al., *Molecular Cloning A Laboratory Manual*, 2nd Ed., Vols. 1-3, Cold Springs Harbor Press (1989); and Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Cold Spring Harbor, New York (1982), all of which are incorporated by reference herein. For example, conditions such as temperature, concentration of components, hybridization and washing times, buffer components, and their pH and ionic strength may be varied.

#### 6.7 Nucleic Acids of the Invention

The sequences falling within the scope of the present invention are not limited to the specific sequences herein described, but also include allelic variations thereof. Allelic variations can be routinely determined by comparing the sequence provided in SEQ ID NOs:1-2, a representative fragment thereof, or a nucleotide sequence at least 99.9% identical to SEQ ID NO:1-2 with a sequence from another isolate of the same species. Furthermore, to accommodate codon variability, the invention includes nucleic acid molecules coding for the same amino acid sequences as do the specific ORFs disclosed

herein. In other words, in the coding region of an ORF, substitution of one codon for another which encodes the same amino acid is expressly contemplated.

5 Any specific sequence disclosed herein can be readily screened for errors by resequencing a particular fragment, such as an ORF, in both directions (i.e., sequence both strands).

The present invention further provides recombinant constructs comprising a nucleic acid having the sequence of any one of SEQ ID NOs: 1-2 or a fragment thereof. The recombinant constructs of the present invention  
10 comprise a vector, such as a plasmid or viral vector, into which a nucleic acid having the sequence of any one of SEQ ID NOs 1-2 or a fragment thereof is inserted, in a forward or reverse orientation. In the case of a vector comprising one of the ORFs of the present invention, the vector may further comprise regulatory sequences, including for example, a promoter, operably linked to the  
15 ORF. For vectors comprising the EMFs and UMFs of the present invention, the vector may further comprise a marker sequence or heterologous ORF operably linked to the EMF or UMF. Large numbers of suitable vectors and promoters are known to those of skill in the art and are commercially available for generating the recombinant constructs of the present invention. The following vectors are  
20 provided by way of example. Bacterial: pBs, phagescript, PsiX174, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene); pTrc99A, pKK223-3, pKK233-3, pDR540, pRIT5 (Pharmacia). Eukaryotic: pWLneo, pSV2cat, pOG44, PXTI, pSG (Stratagene) pSVK3, pBPV, pMSG, pSVL (Pharmacia).

25 Promoter regions can be selected from any desired gene using CAT (chloramphenicol transferase) vectors or other vectors with selectable markers. Two appropriate vectors are pKK232-8 and pCM7. Particular named bacterial promoters include lacI, lacZ, T3, T7, gpt, lambda PR, and trc. Eukaryotic promoters include CMV immediate early, HSV thymidine kinase, early  
30 and late SV40, LTRs from retrovirus, and mouse metallothionein-I. Selection of

the appropriate vector and promoter is well within the level of ordinary skill in the art.

Generally, recombinant expression vectors will include origins of replication and selectable markers permitting transformation of the host cell, e.g., the ampicillin resistance gene of *E. coli* and *S. cerevisiae* TRP1 gene, and a promoter derived from a highly-expressed gene to direct transcription of a downstream structural sequence. Such promoters can be derived from operons encoding glycolytic enzymes such as 3-phosphoglycerate kinase (PGK), a-factor, acid phosphatase, or heat shock proteins, among others. The heterologous structural sequence is assembled in appropriate phase with translation initiation and termination sequences, and preferably, a leader sequence capable of directing secretion of translated protein into the periplasmic space or extracellular medium. Optionally, the heterologous sequence can encode a fusion protein including an N-terminal identification peptide imparting desired characteristics, e.g., stabilization or simplified purification of expressed recombinant product.

Useful expression vectors for bacterial use are constructed by inserting a structural DNA sequence encoding a desired protein together with suitable translation initiation and termination signals in operable reading phase with a functional promoter. The vector will comprise one or more phenotypic selectable markers and an origin of replication to ensure maintenance of the vector and to, if desirable, provide amplification within the host. Suitable prokaryotic hosts for transformation include *E. coli*, *Bacillus subtilis*, *Salmonella typhimurium* and various species within the genera *Pseudomonas*, *Streptomyces*, and *Staphylococcus*, although others may also be employed as a matter of choice.

As a representative but nonlimiting example, useful expression vectors for bacterial use can comprise a selectable marker and bacterial origin of replication derived from commercially available plasmids comprising genetic elements of the well known cloning vector pBR322 (ATCC 37017). Such

commercial vectors include, for example, pKK223-3 (Pharmacia Fine Chemicals, Uppsala, Sweden) and GEM 1 (Promega Biotec, Madison, WI, USA). These pBR322 "backbone" sections are combined with an appropriate promoter and the structural sequence to be expressed.

5                   Following transformation of a suitable host strain and growth of the host strain to an appropriate cell density, the selected promoter is induced or derepressed by appropriate means (e.g., temperature shift or chemical induction) and cells are cultured for an additional period. Cells are typically harvested by centrifugation, disrupted by physical or chemical means, and the  
10                   resulting crude extract retained for further purification.

                  Included within the scope of the nucleic acid sequences of the invention are nucleic acid sequences that hybridize under stringent conditions to a fragment of the DNA sequences in Figure 1, which fragment is greater than about 10 bp, preferably 20-50 bp, and even greater than 100 bp.

15                   In accordance with the invention, polynucleotide sequences which encode the novel nucleic acids, or functional equivalents thereof, may be used to generate recombinant DNA molecules that direct the expression of that nucleic acid, or a functional equivalent thereof, in appropriate host cells.

                  The nucleic acid sequences of the invention are further directed  
20                   to sequences which encode variants of the described nucleic acids. These amino acid sequence variants may be prepared by methods known in the art by introducing appropriate nucleotide changes into a native or variant polynucleotide. There are two variables in the construction of amino acid sequence variants: the location of the mutation and the nature of the mutation.  
25                   The amino acid sequence variants of the nucleic acids are preferably constructed by mutating the polynucleotide to give an amino acid sequence that does not occur in nature. These amino acid alterations can be made at sites that differ in the nucleic acids from different species (variable positions) or in highly conserved regions (constant regions). Sites at such locations will  
30                   typically be modified in series, e.g., by substituting first with conservative

choices (e.g., hydrophobic amino acid to a different hydrophobic amino acid) and then with more distant choices (e.g., hydrophobic amino acid to a charged amino acid), and then deletions or insertions may be made at the target site.

Amino acid sequence deletions generally range from about 1 to 30 residues, preferably about 1 to 10 residues, and are typically contiguous. Amino acid insertions include amino- and/or carboxyl-terminal fusions ranging in length from one to one hundred or more residues, as well as intrasequence insertions of single or multiple amino acid residues. Intrasequence insertions may range generally from about 1 to 10 amino residues, preferably from 1 to 5 residues. Examples of terminal insertions include the heterologous signal sequences necessary for secretion or for intracellular targeting in different host cells.

In a preferred method, polynucleotides encoding the novel nucleic acids are changed via site-directed mutagenesis. This method uses oligonucleotide sequences that encode the polynucleotide sequence of the desired amino acid variant, as well as a sufficient adjacent nucleotide on both sides of the changed amino acid to form a stable duplex on either side of the site of being changed. In general, the techniques of site-directed mutagenesis are well known to those of skill in the art and this technique is exemplified by publications such as, Edelman et al., DNA 2:183 (1983). A versatile and efficient method for producing site-specific changes in a polynucleotide sequence was published by Zoller and Smith, Nucleic Acids Res. 10:6487-6500 (1982).

PCR may also be used to create amino acid sequence variants of the novel nucleic acids. When small amounts of template DNA are used as starting material, primer(s) that differs slightly in sequence from the corresponding region in the template DNA can generate the desired amino acid variant. PCR amplification results in a population of product DNA fragments that differ from the polynucleotide template encoding the polypeptide at the position specified by the primer. The product DNA fragments replace the corresponding region in the plasmid and this gives the desired amino acid variant.



A further technique for generating amino acid variants is the cassette mutagenesis technique described in Wells et al., *Gene* 34:315 (1985); and other mutagenesis techniques well known in the art, such as, for example, the techniques in Sambrook et al., *supra*, and *Current Protocols in Molecular Biology*, Ausubel et al.

Due to the inherent degeneracy of the genetic code, other DNA sequences which encode substantially the same or a functionally equivalent amino acid sequence may be used in the practice of the invention for the cloning and expression of these novel nucleic acids. Such DNA sequences include those which are capable of hybridizing to the appropriate novel nucleic acid sequence under stringent conditions.

Furthermore, knowledge of the DNA sequence provided by the present invention allows for the modification of cells to permit, or increase, expression of endogenous CD39-like polypeptides. Cells can be modified (e.g., by homologous recombination) to provide increased CD39-like expression by replacing, in whole or in part, the naturally occurring CD39-like promoter with all or part of a heterologous promoter so that the cells express CD39-like polypeptides at a higher level. The heterologous promoter is inserted in such a manner that it is operatively linked to CD39-like encoding sequences. See, for example, PCT International Publication No. WO94/12650, PCT International Publication No. WO92/20808, and PCT International Publication No. WO91/09955. It is also contemplated that, in addition to heterologous promoter DNA, amplifiable marker DNA (e.g., *ada*, *dhfr*, and the multifunctional CAD gene which encodes carbamyl phosphate synthase, aspartate transcarbamylase, and dihydroorotase) and/or intron DNA may be inserted along with the heterologous promoter DNA. If linked to the CD39-like coding sequence, amplification of the marker DNA by standard selection methods results in co-amplification of the CD39-like coding sequences in the cells.

The polynucleotides of the present invention also make possible the development, through, e.g., homologous recombination or knock out

strategies, of animals that fail to express functional CD39-L4 or that express a variant of CD39-L4. Such animals are useful as models for studying the in vivo activities of CD39-L4 as well as for studying modulators of CD39-L4.

## 5        **6.8 Identification of Polymorphisms**

Polymorphisms can be identified in a variety of ways known in the art which all generally involve obtaining a sample from a patient, analyzing DNA from the sample, optionally involving isolation or amplification of the DNA, and identifying the presence of the polymorphism in the DNA. For example, PCR may be used to amplify an appropriate fragment of genomic DNA which may then be sequenced. Alternatively, the DNA may be subjected to allele-specific oligonucleotide hybridization (in which appropriate oligonucleotides are hybridized to the DNA under conditions permitting detection of a single base mismatch) or to a single nucleotide extension assay (in which an oligonucleotide that hybridizes immediately adjacent to the position of the polymorphism is extended with one or more labelled nucleotides). In addition, traditional restriction fragment length polymorphism analysis (using restriction enzymes that provide differential digestion of the genomic DNA depending on the presence or absence of the polymorphism) may be performed.

Alternatively, a polymorphism resulting in a change in the amino acid sequence could also be detected by detecting a corresponding change in amino acid sequence of the protein, e.g., by an antibody specific to the variant sequence.

## 25        **6.9 Hosts**

The present invention further provides host cells containing SEQ ID NOs:1-2 of the present invention, wherein the nucleic acid has been introduced into the host cell using known transformation, transfection or infection methods. The host cell can be a higher eukaryotic host cell, such as a mammalian cell, a lower eukaryotic host cell, such as a yeast cell, or the host

cell can be a prokaryotic cell, such as a bacterial cell. Introduction of the recombinant construct into the host cell can be effected by calcium phosphate transfection, DEAE, dextran mediated transfection, or electroporation (Davis, L. et al., Basic Methods in Molecular Biology (1986)).

5 The host cells containing one of SEQ ID NOs:1-2 of the present invention, can be used in conventional manners to produce the gene product encoded by the isolated fragment (in the case of an ORF) or can be used to produce a heterologous protein under the control of the EMF.

10 Any host/vector system can be used to express one or more of the ORFs of the present invention. These include, but are not limited to, eukaryotic hosts such as HeLa cells, Cv-1 cell, COS cells, and Sf9 cells, as well as prokaryotic host such as E. coli and B. subtilis. The most preferred cells are those which do not normally express the particular polypeptide or protein or which expresses the polypeptide or protein at low natural level.

15 Mature proteins can be expressed in mammalian cells, yeast, bacteria, or other cells under the control of appropriate promoters. Cell-free translation systems can also be employed to produce such proteins using RNAs derived from the DNA constructs of the present invention. Appropriate cloning and expression vectors for use with prokaryotic and eukaryotic hosts are described by Sambrook, et al., in Molecular Cloning: A Laboratory Manual, Second Edition, Cold Spring Harbor, New York (1989), the disclosure of which is hereby incorporated by reference.

20 Various mammalian cell culture systems can also be employed to express recombinant protein. Examples of mammalian expression systems include the COS-7 lines of monkey kidney fibroblasts, described by Gluzman, Cell 23:175 (1981), and other cell lines capable of expressing a compatible vector, for example, the C127, 3T3, CHO, HeLa and BHK cell lines. Mammalian expression vectors will comprise an origin of replication, a suitable promoter and also any necessary ribosome binding sites, polyadenylation site, splice donor and acceptor sites, transcriptional termination sequences, and 5' flanking

nontranscribed sequences. DNA sequences derived from the SV40 viral genome, for example, SV40 origin, early promoter, enhancer, splice, and polyadenylation sites may be used to provide the required nontranscribed genetic elements.

Recombinant polypeptides and proteins produced in bacterial culture are usually isolated by initial extraction from cell pellets, followed by one or more salting-out, aqueous ion exchange or size exclusion chromatography steps. Protein refolding steps can be used, as necessary, in completing configuration of the mature protein. Finally, high performance liquid chromatography (HPLC) can be employed for final purification steps. Microbial cells employed in expression of proteins can be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or use of cell lysing agents.

#### 6.10 Peptides

The present invention further provides isolated polypeptides encoded by the nucleic acid fragments of the present invention or by degenerate variants of the nucleic acid fragments of the present invention. By "degenerate variant" is intended nucleotide fragments which differ from a nucleic acid fragment of the present invention (e.g., an ORF) by nucleotide sequence but, due to the degeneracy of the Genetic Code, encode an identical polypeptide sequence. Preferred nucleic acid fragments of the present invention are the ORFs which encode proteins.

A variety of methodologies known in the art can be utilized to obtain any one of the isolated polypeptides or proteins of the present invention. At the simplest level, the amino acid sequence can be synthesized using commercially available peptide synthesizers. This is particularly useful in producing small peptides and fragments of larger polypeptides. Fragments are useful, for example, in generating antibodies against the native polypeptide. In an alternative method, the polypeptide or protein is purified from bacterial cells

which naturally produce the polypeptide or protein. One skilled in the art can readily follow known methods for isolating polypeptides and proteins in order to obtain one of the isolated polypeptides or proteins of the present invention. These include, but are not limited to, immunochromatography, HPLC, size-exclusion chromatography, ion-exchange chromatography, and immuno-affinity chromatography. See, e.g., Scopes, *Protein Purification: Principles and Practice*, Springer-Verlag (1994); Sambrook, et al., in *Molecular Cloning: A Laboratory Manual*; Ausubel et al., *Current Protocols in Molecular Biology*.

The polypeptides and proteins of the present invention can alternatively be purified from cells which have been altered to express the desired polypeptide or protein. As used herein, a cell is said to be altered to express a desired polypeptide or protein when the cell, through genetic manipulation, is made to produce a polypeptide or protein which it normally does not produce or which the cell normally produces at a lower level. One skilled in the art can readily adapt procedures for introducing and expressing either recombinant or synthetic sequences into eukaryotic or prokaryotic cells in order to generate a cell which produces one of the polypeptides or proteins of the present invention.

The purified polypeptides are used in *in vitro* binding assays which are well known in the art to identify molecules which bind to the polypeptides. These molecules include but are not limited to, for e.g., small molecules, molecules from combinatorial libraries, antibodies or other proteins. The molecules identified in the binding assay are then tested for antagonist or agonist activity in *in vivo* tissue culture or animal models that are well known in the art. In brief, the molecules are titrated into a plurality of cell cultures or animals and then tested for either cell/animal death or prolonged survival of the animal/cells.

In addition, the binding molecules may be complexed with toxins, e.g., ricin or cholera, or with other compounds that are toxic to cells. The

toxin-binding molecule complex is then targeted to the tumor or other cell by the specificity of the binding molecule for SEQ ID NOs:3-4.

### 6.11 Gene Therapy

5 Mutations in the CD39-like gene that result in loss of normal function of the CD39-like gene product underlie CD39-related human disease states. The invention comprehends gene therapy to restore CD39-like activity that would thus be indicated in treating those disease states. Delivery of a functional CD39-like gene to appropriate cells is effected *ex vivo*, *in situ*, or *in vivo* by use of vectors, and more particularly viral vectors (e.g., adenovirus, adeno-associated virus, or a retrovirus), or *ex vivo* by use of physical DNA transfer methods (e.g., liposomes or chemical treatments). See, for example, Anderson, Nature, supplement to vol. 392, no 6679, pp. 25-30 (1998). For additional reviews of gene therapy technology, see Friedmann, Science, 244: 1275-1281 (1989); Verma, Scientific American: 68-84 (1990); and Miller, Nature, 357: 455-460 (1992). Alternatively, it is contemplated that in other human disease states, preventing the expression of or inhibiting the activity of CD39-like polypeptides will be useful in treating the disease states. It is contemplated that antisense therapy or gene therapy could be applied to negatively regulate the expression of CD39-like polypeptides.

### 6.12 Antibodies

In general, techniques for preparing polyclonal and monoclonal antibodies as well as hybridomas capable of producing the desired antibody are well known in the art (Campbell, A.M., Monoclonal Antibodies Technology: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1984); St. Groth et al., J. Immunol. 35:1-21 (1990); Kohler and Milstein, Nature 256:495-497 (1975)), the trioma technique, the human B-cell hybridoma technique (Kozbor et al., Immunology

Today 4:72 (1983); Cole et al., in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985), pp. 77-96).

Any animal (mouse, rabbit, etc.) which is known to produce antibodies can be immunized with a peptide or polypeptide of the invention. Methods for immunization are well known in the art. Such methods include subcutaneous or intraperitoneal injection of the polypeptide. One skilled in the art will recognize that the amount of the protein encoded by the ORF of the present invention used for immunization will vary based on the animal which is immunized, the antigenicity of the peptide and the site of injection.

The protein which is used as an immunogen may be modified or administered in an adjuvant in order to increase the protein's antigenicity. Methods of increasing the antigenicity of a protein are well known in the art and include, but are not limited to, coupling the antigen with a heterologous protein (such as globulin or  $\alpha$ -galactosidase) or through the inclusion of an adjuvant during immunization.

For monoclonal antibodies, spleen cells from the immunized animals are removed, fused with myeloma cells, such as SP2/0-Ag14 myeloma cells, and allowed to become monoclonal antibody producing hybridoma cells.

Any one of a number of methods well known in the art can be used to identify the hybridoma cell which produces an antibody with the desired characteristics. These include screening the hybridomas with an ELISA assay, western blot analysis, or radioimmunoassay (Lutz et al., *Exp. Cell Research*. 175:109-124 (1988)).

Hybridomas secreting the desired antibodies are cloned and the class and subclass is determined using procedures known in the art (Campbell, A.M., *Monoclonal Antibody Technology: Laboratory Techniques in Biochemistry and Molecular Biology*, Elsevier Science Publishers, Amsterdam, The Netherlands (1984)).

Techniques described for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce single chain antibodies to proteins of the present invention.

For polyclonal antibodies, antibody containing antiserum is isolated from the immunized animal and is screened for the presence of antibodies with the desired specificity using one of the above-described procedures.

The present invention further provides the above-described antibodies in detectably labeled form. Antibodies can be detectably labeled through the use of radioisotopes, affinity labels (such as biotin, avidin, etc.), enzymatic labels (such as horseradish peroxidase, alkaline phosphatase, etc.) fluorescent labels (such as FITC or rhodamine, etc.), paramagnetic atoms, etc. Procedures for accomplishing such labeling are well-known in the art, for example, see (Sternberger, L.A. et al., J. Histochem. Cytochem. 18:315 (1970); Bayer, E.A. et al., Meth. Enzym. 62:308 (1979); Engval, E. et al., Immunol. 109:129 (1972); Goding, J.W. J. Immunol. Meth. 13:215 (1976)).

The labeled antibodies of the present invention can be used for in vitro, in vivo, and in situ assays to identify cells or tissues in which a fragment of the polypeptide of interest is expressed. The antibodies may also be used directly in therapies or other diagnostics.

The present invention further provides the above-described antibodies immobilized on a solid support. Examples of such solid supports include plastics such as polycarbonate, complex carbohydrates such as agarose and sepharose, acrylic resins and such as polyacrylamide and latex beads. Techniques for coupling antibodies to such solid supports are well known in the art (Weir, D.M. et al., "Handbook of Experimental Immunology" 4th Ed., Blackwell Scientific Publications, Oxford, England, Chapter 10 (1986); Jacoby, W.D. et al., Meth. Enzym. 34 Academic Press, N.Y. (1974)). The immobilized antibodies of the present invention can be used for in vitro, in vivo, and in situ assays as well as for immuno-affinity purification of the proteins of the present invention.



### 6.13 Computer Readable Sequences

In one application of this embodiment, a nucleotide sequence of the present invention can be recorded on computer readable media. As used herein, "computer readable media" refers to any medium which can be read and accessed directly by a computer. Such media include, but are not limited to: magnetic storage media, such as floppy discs, hard disc storage medium, and magnetic tape; optical storage media such as CD-ROM; electrical storage media such as RAM and ROM; and hybrids of these categories such as magnetic/optical storage media. A skilled artisan can readily appreciate how any of the presently known computer readable mediums can be used to create a manufacture comprising computer readable medium having recorded thereon a nucleotide sequence of the present invention.

As used herein, "recorded" refers to a process for storing information on computer readable medium. A skilled artisan can readily adopt any of the presently known methods for recording information on computer readable medium to generate manufactures comprising the nucleotide sequence information of the present invention.

A variety of data storage structures are available to a skilled artisan for creating a computer readable medium having recorded thereon a nucleotide sequence of the present invention. The choice of the data storage structure will generally be based on the means chosen to access the stored information. In addition, a variety of data processor programs and formats can be used to store the nucleotide sequence information of the present invention on computer readable medium. The sequence information can be represented in a word processing text file, formatted in commercially-available software such as WordPerfect and Microsoft Word, or represented in the form of an ASCII file, stored in a database application, such as DB2, Sybase, Oracle, or the like. A skilled artisan can readily adapt any number of dataprocessor structuring formats (e.g. text file or database) in order to obtain computer readable medium having recorded thereon the nucleotide sequence information of the present invention.

By providing the nucleotide sequence of SEQ ID NOs:1-2, a representative fragment thereof, or a nucleotide sequence at least 99.9% identical to SEQ ID NOs:1-2 in computer readable form, a skilled artisan can routinely access the sequence information for a variety of purposes. Computer software is publicly available which allows a skilled artisan to access sequence information provided in a computer readable medium. The examples which follow demonstrate how software which implements the BLAST (Altschul et al., J. Mol. Biol. 215:403-410 (1990)) and BLAZE (Brutlag et al., Comp. Chem. 17:203-207 (1993)) search algorithms on a Sybase system is used to identify open reading frames (ORFs) within a nucleic acid sequence. Such ORFs may be protein encoding fragments and may be useful in producing commercially important proteins such as enzymes used in fermentation reactions and in the production of commercially useful metabolites.

As used herein, "a computer-based system" refers to the hardware means, software means, and data storage means used to analyze the nucleotide sequence information of the present invention. The minimum hardware means of the computer-based systems of the present invention comprises a central processing unit (CPU), input means, output means, and data storage means. A skilled artisan can readily appreciate that any one of the currently available computer-based systems are suitable for use in the present invention.

As stated above, the computer-based systems of the present invention comprise a data storage means having stored therein a nucleotide sequence of the present invention and the necessary hardware means and software means for supporting and implementing a search means. As used herein, "data storage means" refers to memory which can store nucleotide sequence information of the present invention, or a memory access means which can access manufactures having recorded thereon the nucleotide sequence information of the present invention.

As used herein, "search means" refers to one or more programs which are implemented on the computer-based system to compare a target

sequence or target structural motif with the sequence information stored within the data storage means. Search means are used to identify fragments or regions of a known sequence which match a particular target sequence or target motif. A variety of known algorithms are disclosed publicly and a variety of commercially available software for conducting search means are and can be used in the computer-based systems of the present invention. Examples of such software includes, but is not limited to, MacPattern (EMBL), BLASTN and BLASTA (NPOLYPEPTIDEIA). A skilled artisan can readily recognize that any one of the available algorithms or implementing software packages for conducting homology searches can be adapted for use in the present computer-based systems.

As used herein, a "target sequence" can be any nucleic acid or amino acid sequence of six or more nucleotides or two or more amino acids. A skilled artisan can readily recognize that the longer a target sequence is, the less likely a target sequence will be present as a random occurrence in the database. The most preferred sequence length of a target sequence is from about 10 to 100 amino acids or from about 30 to 300 nucleotide residues. However, it is well recognized that searches for commercially important fragments, such as sequence fragments involved in gene expression and protein processing, may be of shorter length.

As used herein, "a target structural motif," or "target motif," refers to any rationally selected sequence or combination of sequences in which the sequence(s) are chosen based on a three-dimensional configuration which is formed upon the folding of the target motif. There are a variety of target motifs known in the art. Protein target motifs include, but are not limited to, enzyme active sites and signal sequences. Nucleic acid target motifs include, but are not limited to, promoter sequences, hairpin structures and inducible expression elements (protein binding sequences).

## 6.14 Expression Modulating Sequences

EMF sequences can be identified within a genome by their proximity to the ORFs. An intergenic segment, or a fragment of the intergenic segment, from about 10 to 200 nucleotides in length, taken 5' from any ORF will modulate the expression of an operably linked 3' ORF in a fashion similar to that found with the naturally linked ORF sequence. As used herein, an "intergenic segment" refers to the fragments of a genome which are between two ORF(S) herein described. Alternatively, EMFs can be identified using known EMFs as a target sequence or target motif in the computer-based systems of the present invention.

The presence and activity of an EMF can be confirmed using an EMF trap vector. An EMF trap vector contains a cloning site 5' to a marker sequence. A marker sequence encodes an identifiable phenotype, such as antibiotic resistance or a complementing nutrition auxotrophic factor, which can be identified or assayed when the EMF trap vector is placed within an appropriate host under appropriate conditions. As described above, an EMF will modulate the expression of an operably linked marker sequence. A more detailed discussion of various marker sequences is provided below.

A sequence which is suspected as being an EMF is cloned in all three reading frames in one or more restriction sites upstream from the marker sequence in the EMF trap vector. The vector is then transformed into an appropriate host using known procedures and the phenotype of the transformed host is examined under appropriate conditions. As described above, an EMF will modulate the expression of an operably linked marker sequence.

## 6.15 Triplex Helix Formation

In addition, the fragments of the present invention, as broadly described, can be used to control gene expression through triple helix formation or antisense DNA or RNA, both of which methods are based on the binding of a polynucleotide sequence to DNA or RNA. Polynucleotides suitable for use in

these methods are usually 20 to 40 bases in length and are designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee et al., Nucl. Acids Res. 6:3073 (1979); Cooney et al., Science 15241:456 (1988); and Dervan et al., Science 251:1360 (1991)) or to the mRNA itself (antisense - Olmno, J. Neurochem. 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)).

Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in model systems. Information contained in the sequences of the present invention is necessary for the design of an antisense or triple helix oligonucleotide.

#### 6.16 Diagnostic Assays and Kits

The present invention further provides methods to identify the expression of one of the ORFs of the present invention, or homolog thereof, in a test sample, using a nucleic acid probe or antibodies of the present invention.

In detail, such methods comprise incubating a test sample with one or more of the antibodies or one or more of nucleic acid probes of the present invention and assaying for binding of the nucleic acid probes or antibodies to components within the test sample.

Conditions for incubating a nucleic acid probe or antibody with a test sample vary. Incubation conditions depend on the format employed in the assay, the detection methods employed, and the type and nature of the nucleic acid probe or antibody used in the assay. One skilled in the art will recognize that any one of the commonly available hybridization, amplification or immunological assay formats can readily be adapted to employ the nucleic acid probes or antibodies of the present invention. Examples of such assays can be found in Chard, T., An Introduction to Radioimmunoassay and Related Techniques, Elsevier Science Publishers, Amsterdam, The Netherlands (1986);

Bullock, G.R. et al., Techniques in Immunocytochemistry, Academic Press, Orlando, FL Vol. 1 (1982), Vol. 2 (1983), Vol. 3 (1985); Tijssen, P., Practice and Theory of immunoassays: Laboratory Techniques in Biochemistry and Molecular Biology, Elsevier Science Publishers, Amsterdam, The Netherlands (1985).

The test samples of the present invention include cells, protein or membrane extracts of cells, or biological fluids such as sputum, blood, serum, plasma, or urine. The test sample used in the above-described method will vary based on the assay format, nature of the detection method and the tissues, cells or extracts used as the sample to be assayed. Methods for preparing protein extracts or membrane extracts of cells are well known in the art and can be readily be adapted in order to obtain a sample which is compatible with the system utilized.

In another embodiment of the present invention, kits are provided which contain the necessary reagents to carry out the assays of the present invention.

Specifically, the invention provides a compartment kit to receive, in close confinement, one or more containers which comprises: (a) a first container comprising one of the probes or antibodies of the present invention; and (b) one or more other containers comprising one or more of the following: wash reagents, reagents capable of detecting presence of a bound probe or antibody.

In detail, a compartment kit includes any kit in which reagents are contained in separate containers. Such containers include small glass containers, plastic containers or strips of plastic or paper. Such containers allows one to efficiently transfer reagents from one compartment to another compartment such that the samples and reagents are not cross-contaminated, and the agents or solutions of each container can be added in a quantitative fashion from one compartment to another. Such containers will include a container which will accept the test sample, a container which contains the

antibodies used in the assay, containers which contain wash reagents (such as phosphate buffered saline, Tris-buffers, etc.), and containers which contain the reagents used to detect the bound antibody or probe.

Types of detection reagents include labeled nucleic acid probes, labeled secondary antibodies, or in the alternative, if the primary antibody is labeled, the enzymatic, or antibody binding reagents which are capable of reacting with the labeled antibody. One skilled in the art will readily recognize that the disclosed probes and antibodies of the present invention can be readily incorporated into one of the established kit formats which are well known in the art.

#### 6.17 Screening Assays

Using the isolated proteins of the present invention, the present invention further provides methods of obtaining and identifying agents which bind to a protein encoded by one of the ORFs from a nucleic acid with a sequence of one of SEQ ID NOs:1-2, or to a nucleic acid with a sequence of one of SEQ ID NOs:1-2.

In detail, said method comprises the steps of: (a) contacting an agent with an isolated protein encoded by one of the ORFs of the present invention, or nucleic acid of the invention; and (b) determining whether the agent binds to said protein or said nucleic acid.

The agents screened in the above assay can be, but are not limited to, peptides, carbohydrates, vitamin derivatives, or other pharmaceutical agents. The agents can be selected and screened at random or rationally selected or designed using protein modeling techniques.

For random screening, agents such as peptides, carbohydrates, pharmaceutical agents and the like are selected at random and are assayed for their ability to bind to the protein encoded by the ORF of the present invention.

Alternatively, agents may be rationally selected or designed. As used herein, an agent is said to be "rationally selected or designed" when the

agent is chosen based on the configuration of the particular protein. For example, one skilled in the art can readily adapt currently available procedures to generate peptides, pharmaceutical agents and the like capable of binding to a specific peptide sequence in order to generate rationally designed antipeptide peptides, for example see Hurby et al., Application of Synthetic Peptides: Antisense Peptides," In Synthetic Peptides, A User's Guide, W.H. Freeman, NY (1992), pp. 289-307, and Kaspiczak et al., Biochemistry 28:9230-8 (1989), or pharmaceutical agents, or the like.

In addition to the foregoing, one class of agents of the present invention, as broadly described, can be used to control gene expression through binding to one of the ORFs or EMFs of the present invention. As described above, such agents can be randomly screened or rationally designed/selected. Targeting the ORF or EMF allows a skilled artisan to design sequence specific or element specific agents, modulating the expression of either a single ORF or multiple ORFs which rely on the same EMF for expression control.

One class of DNA binding agents are agents which contain base residues which hybridize or form a triple helix formation by binding to DNA or RNA. Such agents can be based on the classic phosphodiester, ribonucleic acid backbone, or can be a variety of sulfhydryl or polymeric derivatives which have base attachment capacity.

Agents suitable for use in these methods usually contain 20 to 40 bases and are designed to be complementary to a region of the gene involved in transcription (triple helix - see Lee et al., Nucl. Acids Res. 6:3073 (1979); Cooney et al., Science 241:456 (1988); and Dervan et al., Science 251:1360 (1991)) or to the mRNA itself (antisense - Okano, J. Neurochem. 56:560 (1991); Oligodeoxynucleotides as Antisense Inhibitors of Gene Expression, CRC Press, Boca Raton, FL (1988)). Triple helix- formation optimally results in a shut-off of RNA transcription from DNA, while antisense RNA hybridization blocks translation of an mRNA molecule into polypeptide. Both techniques have been demonstrated to be effective in model systems. Information contained in the



sequences of the present invention is necessary for the design of an antisense or triple helix oligonucleotide and other DNA binding agents.

Agents which bind to a protein encoded by one of the ORFs of the present invention can be used as a diagnostic agent, in the control of bacterial infection by modulating the activity of the protein encoded by the ORF. Agents which bind to a protein encoded by one of the ORFs of the present invention can be formulated using known techniques to generate a pharmaceutical composition.

#### 6.18 Use of Nucleic Acids as Probes

Another aspect of the subject invention is to provide for polypeptide-specific nucleic acid hybridization probes capable of hybridizing with naturally occurring nucleotide sequences. The hybridization probes of the subject invention may be derived from the nucleotide sequence of the SEQ ID NOs:1-2. Because the corresponding gene is expressed in only one out of 18 tissues tested, namely macrophages, a hybridization probe derived from SEQ ID NOs:1-2 can be used as an indicator of the presence of macrophage RNA in a sample. Any suitable hybridization technique can be employed, such as, for example, in situ hybridization.

PCR as described US Patent Nos 4,683,195 and 4,965,188 provides additional uses for oligonucleotides based upon the nucleotide sequences. Such probes used in PCR may be of recombinant origin, may be chemically synthesized, or a mixture of both. The probe will comprise a discrete nucleotide sequence for the detection of identical sequences or a degenerate pool of possible sequences for identification of closely related genomic sequences.

Other means for producing specific hybridization probes for nucleic acids include the cloning of nucleic acid sequences into vectors for the production of mRNA probes. Such vectors are known in the art and are commercially available and may be used to synthesize RNA probes in vitro by

means of the addition of the appropriate RNA polymerase as T7 or SP6 RNA polymerase and the appropriate radioactively labeled nucleotides.

The nucleotide sequences may be used to construct hybridization probes for mapping their respective genomic sequences. The nucleotide sequence provided herein may be mapped to a chromosome or specific regions of a chromosome using well known genetic and/or chromosomal mapping techniques. These techniques include in situ hybridization, linkage analysis against known chromosomal markers, hybridization screening with libraries or flow-sorted chromosomal preparations specific to known chromosomes, and the like. The technique of fluorescent in situ hybridization of chromosome spreads has been described, among other places, in Verma et al (1988) Human Chromosomes: A Manual of Basic Techniques, Pergamon Press, New York NY. Fluorescent in situ hybridization of chromosomal preparations and other physical chromosome mapping techniques may be correlated with additional genetic map data. Examples of genetic map data can be found in the 1994 Genome Issue of Science (265:1981f). Correlation between the location of a nucleic acid on a physical chromosomal map and a specific disease (or predisposition to a specific disease) may help delimit the region of DNA associated with that genetic disease. The nucleotide sequences of the subject invention may be used to detect differences in gene sequences between normal, carrier or affected individuals.

The nucleotide sequence may be used to produce purified polypeptides using well known methods of recombinant DNA technology. Among the many publications that teach methods for the expression of genes after they have been isolated is Goeddel (1990) Gene Expression Technology, Methods and Enzymology, Vol 185, Academic Press, San Diego. Polypeptides may be expressed in a variety of host cells, either prokaryotic or eukaryotic. Host cells may be from the same species from which a particular polypeptide nucleotide sequence was isolated or from a different species. Advantages of producing polypeptides by recombinant DNA technology include obtaining

adequate amounts of the protein for purification and the availability of simplified purification procedures.

Each sequence so obtained was compared to sequences in GenBank using a search algorithm developed by Applied Biosystems and incorporated into the INHERIT™ 670 Sequence Analysis System. In this algorithm, Pattern Specification Language (developed by TRW Inc., Los Angeles, CA) was used to determine regions of homology. The three parameters that determine how the sequence comparisons run were window size, window offset, and error tolerance. Using a combination of these three parameters, the DNA database was searched for sequences containing regions of homology to the query sequence, and the appropriate sequences were scored with an initial value. Subsequently, these homologous regions were examined using dot matrix homology plots to distinguish regions of homology from chance matches. Smith-Waterman alignments were used to display the results of the homology search.

Peptide and protein sequence homologies were ascertained using the INHERIT™ 670 Sequence Analysis System in a way similar to that used in DNA sequence homologies. Pattern Specification Language and parameter windows were used to search protein databases for sequences containing regions of homology which were scored with an initial value. Dot-matrix homology plots were examined to distinguish regions of significant homology from chance matches.

Alternatively, BLAST, which stands for Basic Local Alignment Search Tool, is used to search for local sequence alignments (Altschul SF (1993) J Mol Evol 36:290-300; Altschul, SF et al (1990) J Mol Biol 215:403-10). BLAST produces alignments of both nucleotide and amino acid sequences to determine sequence similarity. Because of the local nature of the alignments, BLAST is especially useful in determining exact matches or in identifying homologs. Whereas it is ideal for matches which do not contain gaps, it is

inappropriate for performing motif-style searching. The fundamental unit of BLAST algorithm output is the High-scoring Segment Pair (HSP).

An HSP consists of two sequence fragments of arbitrary but equal lengths whose alignment is locally maximal and for which the alignment score meets or exceeds a threshold or cutoff score set by the user. The BLAST approach is to look for HSPs between a query sequence and a database sequence, to evaluate the statistical significance of any matches found, and to report only those matches which satisfy the user-selected threshold of significance. The parameter E establishes the statistically significant threshold for reporting database sequence matches. E is interpreted as the upper bound of the expected frequency of chance occurrence of an HSP (or set of HSPs) within the context of the entire database search. Any database sequence whose match satisfies E is reported in the program output.

In addition, BLAST analysis was used to search for related molecules within the libraries of the LIFESEQ™ database. This process, an "electronic northern" analysis is analogous to northern blot analysis in that it uses one cellubrevin sequence at a time to search for identical or homologous molecules at a set stringency. The stringency of the electronic northern is based on "product score". The product score is defined as (% nucleotide or amino acid [between the query and reference sequences] in Blast multiplied by the % maximum possible BLAST score [based on the lengths of query and reference sequences]) divided by 100. At a product score of 40, the match will be exact within a 1-2% error; and at 70, the match will be exact. Homologous or related molecules can be identified by selecting those which show product scores between approximately 15 and 30.

#### 6.19 SEQ ID NOs:1-8

Referring to Figure 1, SEQ ID NO:1 is the nucleotide sequence of an expressed sequence tag corresponding to a polynucleotide isolated from a cDNA library of human fetal liver-spleen. SEQ ID NO:2 is an extended version

of SEQ ID NO:1 obtained as described in Example 34, and the encoded polypeptide in SEQ ID NO: 3 is referred to herein as CD39-L66.. SEQ ID NO:2 encodes a polypeptide having the amino acid sequence of SEQ ID NO:3 (shown in Figure 2). The open reading frame corresponding to SEQ ID NO:3 starts at nucleotide 246, as numbered from the 5' end of SEQ ID NO:2. This open reading frame encodes a polypeptide 428 amino acids in length. The estimated molecular weight of the unglycosylated polypeptide is approximately 47.52 kDa.

Protein database searches with the BLAST algorithm indicate that SEQ ID NO:3 is homologous to the CD39 family. Figure 3 shows the amino acid sequence alignment between SEQ ID NO:3 (identified as "246 prot") and human CD39 ("CD39Human.seq"), indicating that the two sequences share 30% amino acid sequence identity. Moreover, a higher degree of homology between the apyrase conserved regions (Kaczmarek et al., J. Biol. Chem. 271:33116-33122 (1996) is observed. In particular, an almost perfect match to a putative ATP-binding region was found from amino acids 54-58, DAGST (DAGSS in CD39). In addition, the DLGGASTQ motif (DLGGASTQ in CD39), which is very well conserved among ATPDases, is found from amino acids 199-206 in SEQ ID NO:3. Other regions conserved in apyrases were found from amino acids 129-134, ATAGLR (ATAGMR in CD39) and from amino acids 169-173, GSDEG (GQEEG in CD39).

SEQ ID NO:3 differs from CD39 in that SEQ ID NO:3 contains a hydrophobic stretch of 22 amino acids at its amino terminus, which is indicative of a leader peptide. SEQ ID NO:3 also lacks the transmembrane domain found at the carboxyl terminus of CD39. These features indicate that SEQ ID NO:3 is a soluble ATPDase.

SEQ ID NO:3 shares an even higher degree of homology (83% identity) with a murine NTPase, as shown in the amino acid sequence alignment presented in Fig. 4 (SEQ ID NO:3 is identified as "246 prot," and mouse CD39 as "mur ntpase").

The message encoding SEQ ID NO:3 is tightly regulated in a tissue-specific manner. An expression study using a semiquantitative PCR/Southern blot approach revealed a significant level of expression in macrophage. In contrast, human CD39 is expressed in tissues such as placenta, lung, skeletal muscle, kidney, and heart.

SEQ ID NO: 4 is the polynucleotide sequence for CD39-L4, described in Chadwick et al., *Genomics*, 50(3):357-67 (1998); SEQ ID NO: 5 is the corresponding amino acid sequence.

SEQ ID NO: 6 is the polynucleotide sequence for a CD39-L4 variant designated ACRIII, wherein the following amino acid substitutions have been made: D168-T, S170-Q and L175-F; SEQ ID NO: 7 is the corresponding amino acid sequence.

SEQ ID NO: 8 is the genomic sequence for the human CD39-L4 gene; exons appear at nucleotides 1-288 (exon 1), 1281-1580 (exon 2), 1820-1855 (exon 3) 2467-2555 (exon 4), 2863-2942 (exon 5), 3889-3950 (exon 6), 4894-4995 (exon 7), 5847-5987 (exon 8), 6966-7138 (exon 9) and 8556-9365 (exon 10).

## 6.20 Uses of Novel CD39-Like Polypeptides and Antibodies

Polypeptides of the invention having ATPDase activity are useful for inhibiting platelet function and can therefore be employed in the prophylaxis or treatment of pathological conditions caused by or involving thrombosis or excessive coagulation or excessive platelet aggregation, such as myocardial infarction, cerebral ischemia, angina, and the like. Polypeptides of the invention can also be used in the maintenance of vascular grafts. Platelet function can be measured by any of a number of standard assays, such as, for example, the platelet aggregation assay described in Example 5.

Such pathological conditions include conditions caused by or involving arterial thrombosis, such as coronary artery thrombosis and resulting myocardial infarction, cerebral artery thrombosis or intracardiac thrombosis (due

to, e.g., atrial fibrillation) and resulting stroke, and other peripheral arterial thrombosis and occlusion; conditions associated with venous thrombosis, such as deep venous thrombosis and pulmonary embolism; conditions associated with exposure of the patient's blood to a foreign or injured tissue surface, including diseased heart valves, mechanical heart valves, vascular grafts, and other extracorporeal devices such as intravascular cannulas, vascular access shunts in hemodialysis patients, hemodialysis machines and cardiopulmonary bypass machines; and conditions associated with coagulopathies, such as hypercoagulability and disseminated intravascular coagulopathy. Co-administration of other agents suitable for treating the pathological condition, e.g., other anti-coagulation agents, is also contemplated.

In particular, variants like the ACRIII mutant described herein are expected to be superior therapeutics for treating such pathological conditions because (1) ACRIII exhibits six-fold greater activity compared to wild type CD39-L4, and (2) ACRIII, like CD39-L4, is uniquely specific for ADP and does not hydrolyze ATP. Thus, adverse side effects from hydrolysis of circulating ATP are avoided.

For instance, ATP is known to act as an extracellular signal in many tissues. In the heart, extracellular ATP modulates ionic processes and contractile function (for review see Burnstock, G., *Neuropharmacology* 36:1127). Recently, it has been shown that extracellular ATP markedly inhibits glucose transport in rat cardiomyocytes (Fisher Y. et al., *J. Biol. Chem.* 274:755-761). Another source of extracellular ATP is that released from parenchymal cells under hypoxic or ischemic conditions (Skobel, E., and Kammermeier, H. *Biochim. Biophys. Acta* 1362:128-134). ATP is also involved in the modulation of anti-IgE-induced release of histamine from human lung mast cells (Schulman E. S., et al., *Am. J. Respir. Cell Mol. Biol.* 20:520-537).

Furthermore, the ability of CD39-L4 to hydrolyze NDPs other than ADP has implications outside the circulatory system. For instance, it has been reported that UDP is the most potent agonist for the human P2Y<sub>6</sub> receptor.

Communi, et al., Bioch Bioph Res Com 222:303-308 (1996). This receptor is expressed in several tissues including infiltrating T cells present in inflammatory bowel disease. Somers, et al., Lab Invest 78:1375-1383 (1998). In this microenvironment, a molecule with the enzymatic properties of CD39-L4 could influence T cell responses by modifying the extracellular half-life of UDP. Another role for CD39-L4 has been suggested by the report that mouse CD39-L4 maps closely to a locus associated with audigenic brain seizures in mice. See Chadwick, et al., Genomics 50:357-367 (1998); Seyfried, et al., Genetics 99:117-126 (1981). This locus, known as Asp-1, is thought to be linked or to correspond to a factor that influences Ca<sup>2+</sup>-ATPase activity. Neumann, et al., Behav. Genetics 20:307-323 (1990).

Additionally, the polypeptides of the invention can be used as molecular weight markers, and as a food supplement. A polypeptide consisting of SEQ ID NO:3, for example, has a molecular mass of approximately 47.52 kD in its unglycosylated form. Protein food supplements are well known and the formulation of suitable food supplements including polypeptides of the invention is within the level of skill in the food preparation art.

The polypeptides of the invention are also useful for making antibody substances that are specifically immunoreactive with CD39-like proteins. Antibodies and portions thereof (e.g., Fab fragments) which bind to the polypeptides of the invention can be used to identify the presence of such polypeptides in a sample. For example, the level of the native protein corresponding to SEQ ID NO:3 in a blood sample can be determined as an indication of vascular condition. Such determinations are carried out using any suitable immunoassay format, and any polypeptide of the invention that is specifically bound by the antibody can be employed as a positive control.

The polypeptides of the invention are administered by any route that delivers an effective dosage to the desired site of action. The determination of a suitable route of administration and an effective dosage for a particular indication is within the level of skill in the art. For treatment of vascular disease,



polypeptides according to the invention are generally administered intravenously. In vivo murine studies with soluble human CD39 have shown that mice injected intravenously with 50 mg recombinant soluble human CD39 in 100 ml sterile saline had biologically active CD39 in their sera for an extended period of time, with an elimination half-life of almost 2 days (Gayle, R.B., et al., J. Clinical Invest. 101:1851-1859 (1998)). Suitable dosage ranges for the polypeptides of the invention can be extrapolated from these dosages or from similar studies in appropriate animal models. Dosages can then be adjusted as necessary by the clinician to provide maximal therapeutic benefit.

The present invention is illustrated in the following examples. Upon consideration of the present disclosure, one of skill in the art will appreciate that many other embodiments and variations may be made in the scope of the present invention. Accordingly, it is intended that the broader aspects of the present invention not be limited to the disclosure of the following examples.

#### EXAMPLE 1

##### Isolation of SEQ ID NO:1 from a cDNA Library of Human Fetal Liver-Spleen

A plurality of novel nucleic acids were obtained from a *b2HFLS20W* cDNA library prepared from human fetal liver-spleen, as described in Bonaldo et al., Genome Res. 6:791-806 (1996), using standard PCR, SBH sequence signature analysis, and Sanger sequencing techniques. The inserts of the library were amplified with PCR using primers specific for vector sequences flanking the inserts. These samples were spotted onto nylon membranes and interrogated with oligonucleotide probes to give sequence signatures. The clones were clustered into groups of similar or identical sequences, and single representative clones were selected from each group for gel sequencing. The 5' sequence of the amplified inserts was then deduced using the reverse M13 sequencing primer in a typical Sanger sequencing

protocol. PCR products were purified and subjected to fluorescent dye terminator cycle sequencing. Single-pass gel sequencing was done using a 377 Applied Biosystems (ABI) sequencer. One of these inserts was identified as a novel sequence not previously obtained from this library and not previously reported in public databases. This sequence is shown in Figure 1 as SEQ ID NO:1.

## EXAMPLE 2

### Isolation of SEQ ID NO:2 and Determination of a Nucleotide Sequence Encoding a 428-Amino Acid Protein with Sequence Homology to CD39

The nucleotide sequence shown in Figure 1, and labeled SEQ ID NO:2, encodes the translated amino acid sequence SEQ ID NO:3, which is shown in Figure 2. The extended nucleotide sequence was obtained by isolating colonies generated from pools of clones from a human macrophage cDNA library (Invitrogen, Cat. # A550-25). Briefly, the macrophage cDNA library was plated on LB/Amp plates (containing 100 mg/ml ampicillin) at a density of about 40,000 colonies/plate. The colonies were lifted onto nitrocellulose filters and hybridized with a radiolabeled probe generated from the original clone (i.e., SEQ ID NO:1).

That the identified clones corresponded to SEQ ID NOs:1 and 2 was confirmed by using gene-specific primers (5'-GCTACCTCACTTCCTTTGAG-3' [SEQ ID NO: 9] and 5'-CTGGCTGGTGAAGTTTTCCTC-3' [SEQ ID NO: 10]) in a PCR-based assay. Then PCR using vector- and gene-specific primers was employed to amplify the 5' portion of the cDNA. Nested primers were used to generate sequence from the amplified product(s). Laser gene™ software was used to edit and "contig" the partial sequences into a full-length sequence. As discussed above, the amino acid sequence has striking homology to CD39, which is involved in modulating platelet reactivity during vascular inflammation. Based in part on the

observed sequence similarity to CD39, the polypeptide encoded by SEQ ID NO: 2 was designated CD39-L66.

### EXAMPLE 3

#### Expression Study Using SEQ ID NO:2

The expression of SEQ ID NO:2 in various tissues was analyzed using a semi-quantitative polymerase chain reaction-based technique. Human cDNA libraries were used as sources of expressed genes from tissues of interest (adult brain, adult heart, adult kidney, adult lymph node, adult liver, adult lung, adult ovary, adult placenta, adult spleen, adult testis, bone marrow, fetal kidney, fetal liver, fetal liver-spleen, fetal skin, fetal brain, fetal leukocyte and macrophage). Gene-specific primers (5'-GCTACCTCACTTCCTTTGAG-3' [SEQ ID NO: 9] and 5'-GCAGGTCTCCAAGGAAGTACG-3' [SEQ ID NO: 11]) were used to amplify portions of the SEQ ID NO:2 sequence from the samples. Amplified products were separated on an agarose gel, transferred and chemically linked to a nylon filter. The filter was then hybridized with a radioactively labeled ( $\alpha^{32}\text{P}$ -dCTP) double-stranded probe generated from the full-length SEQ ID NO:2 sequence using a Klenow polymerase, random-prime method. The filters were washed (high stringency) and used to expose a phosphorimaging screen for several hours. Bands indicated the presence of cDNA including SEQ ID NO:2 sequences in a specific library, and thus mRNA expression in the corresponding cell type or tissue.

Of the 18 human tissues tested, macrophage was the only sample that provided a signal, indicating that expression of SEQ ID NO:2 is tightly regulated. In contrast, the CD39 molecule has been found in tissues such as placenta, lung, skeletal muscle, kidney and heart.

#### EXAMPLE 4

##### Chromosomal Localization of the Gene Corresponding to SEQ ID NOs:1 and 2

Chromosome mapping technologies allow investigators to link genes to specific regions of chromosomes. Assignment to chromosome 14 was performed with the Coriell cell repository monochromosomal panel #2 (NIGMS cell repository). This human rodent somatic cell hybrid panel consists of DNA isolated from 24 hybrid cell cultures retaining 1 human chromosome each. The panel was screened with gene-specific primers (5'-GCTACCTCACTTCCTTGAG-3' [SEQ ID NO: 9] and 5'-CTGGCTGGTGAAGTTTCCTC-3' [SEQ ID NO: 10]) that generated a sequence tag site (STS). The Genebridge 4 radiation hybrid panel was also screened (Research Genetics), and the results of the PCR screening were submitted to the Whitehead/MIT Radiation Hybrid mapping email server at <http://www-genome.wi.mit.edu>.

#### EXAMPLE 5

##### Platelet Aggregation Assay

Blood is anticoagulated with 0.1 volume 3.2% sodium citrate. Platelet-rich plasma (PRP) is prepared with an initial whole blood centrifugation (200 x g, 15 min., 25°C) and a second centrifugation of the PRP (90 x g, 10 min.) to eliminate residual erythrocytes and leukocytes. The stock suspension of PRP is maintained at room temperature under 5% CO<sub>2</sub>-air. The platelet aggregation assay uses a two-sample, four-channel Whole Blood Lumi-Aggregometer, model 560 (Chronolog Corp., Havertown, PA). PRP containing  $1.22 \times 10^8$  platelets is preincubated with the sample to be tested for inhibition of aggregation for 10 min. at 37°C in a siliconized glass cuvette containing a stirring bar, followed by stimulation with either ADP (5 mM), collagen (5 mg/ml), or thrombin (0.1 unit/ml). Platelet aggregation is recorded for at least 10 min. Data are expressed as the percentage of light transmission with platelet-poor plasma equal to 100%.

[illegible][illegible][illegible][illegible]

methyalted parental DNA and then transformed into *E. coli*. Colonies were screened for the insert.

To ascertain whether CD39-L4-6His is secreted, the coding region of the CD39-L4-6His protein was inserted into the pcDNA3.1 expression vector and transiently transfected into COS-7 cells. Cos-7 cells obtained from the American Tissue Type Culture Collection were grown in DMEM supplemented with 10% FBS and 100 units/ml penicillin G and 100  $\mu$ g/ml streptomycin sulfate at 37°C in 10% CO<sub>2</sub>. Transfections were performed at 75% confluency in 10cm plates with Fugene-6 according to the manufacturers instructions. The cells in 7 mls of medium were incubated with 16  $\mu$ l of Fugene-6 and 8  $\mu$ g of DNA for 14-18 hours. At the end of the transfection the medium was replaced with DMEM medium containing low serum (1% FBS). The cells were then incubated for 24-48 hours prior to harvesting.

The CD39-L4-6His was concentrated by treating the cell lysates and medium with Nickel-NTA agarose (Qiagen) followed by SDS/PAGE and immunoblot analysis with an antibody against the Arg-Gly-Ser-6His epitope. Cells were washed twice with PBS containing 0.5  $\mu$ g/ml leupeptin, 0.7  $\mu$ g/ml pepstatin and 0.2  $\mu$ g/ml aprotinin. After a brief sonication and centrifugation step to clear the lysate, the samples were then incubated with a Nickel-NTA resin at 4°C for 2-3 hours. The histidine-tagged protein complexed to the resin was washed three times with PBS before loading onto a 10% SDS/PAGE gel for Western blot analysis. CD39-L4 was detected in both the cell lysate and the medium from cells transfected with the CD39-L4-6His expression vector, but not from control cells. While the predicted molecular weight of CD39-L4-6His is 46 kDa, the immunoreactive protein exhibited a mobility by SDS/PAGE corresponding to a molecular mass of approximately 51 kDa in the media and approximately 48 kDa in the cell lysate. The difference in apparent molecular weight may be due to posttranslational modifications of three potential N-glycosylation sites in the CD39-L4 predicted amino acid sequence.

Secretion of CD39-L4 was also examined by treatment of the transfected cells with brefeldin A, an inhibitor of translocation of secretory proteins from the endoplasmic reticulum to the Golgi apparatus. Chadwick, et al., *Genomics* **50**:357-367 (1998). Brefeldin A was dissolved in ethanol and added to the transfected cells 48 hours after transfection. Both control and brefeldin A treated cells were washed once with PBS and incubated for 8 hours in medium with none or varying dosages of brefeldin A. Increasing dosages of brefeldin A blocked secretion of CD39-L4-6His and led to massive intracellular accumulation.

### Example 7

#### Site-directed mutagenesis of CD39L4

Site directed mutagenesis was employed to increase the enzymatic activity of CD39L4. Amino acid sequence comparisons between CD39 family members reveal four highly homologous regions in all five human members (Chadwick and Frischauf, *Genomics* 50:357-367, 1998). These regions, termed apyrase-conserved regions (ACRs), are present not only in the CD39 family members but other apyrases from species as distant as yeast and plants. Examination of similarities and differences in the CD39 ACRs led to the design of three CD39L4 mutants (see Figure 5). In these mutants, codons encoding CD39 ACR specific residues were used to replace codons from the CD39L4 wild type ACR sequence. Only residues with significantly different structural or chemical properties were replaced. A PCR based approach was used to produce these mutations.

Briefly, the expression vector pCDNA3.1 (Invitrogen) containing the full coding sequence of the CD39L4 gene (with a 6 Histidine tag inserted after Arg 24 in the coding sequence to allow purification of the secreted mature form of the protein) was subjected to a PCR-based site-directed mutagenesis approach using overlapping oligonucleotides [CD39-L4 ACR I mutant (nt 177-148 and 160-204): 5'-GTG AGT GCT CCC TGC ATC TAA CAT AAT TCC-3'

(SEQ ID NO: 12) and 5'-GAT GCA GGG AGC ACT CAC ACT AGT ATT CAT GTT TAC ACC TTT GTG-3' (SEQ ID NO: 13); CD39-L4 ACRII mutant (nt 402-359 and 385-415): 5'-GCG TAG TCC TGC TGT TGC CCC TAG GTA CAC TGG GGT CTT TTT CC-3' (SEQ ID NO: 14) and 5'-GCA ACA GCA GGA CTA CGC TTA CTG CCA GAA C-3' (SEQ ID NO: 15); and CD39-L4 ACR III mutant (nt 532-485 and 513-540): 5'-CCC AAG CGA ATA TGC CTT CGT CTT GTC CAG TCA TGA TGC TAA CAC TGC-3' (SEQ ID NO: 16) and 5'-CGA AGG CAT ATT CGC TTG GGT TAC TGT G-3' (SEQ ID NO:17)]. After amplification of the whole plasmid with Pfu DNA polymerase (Stratagene) (95°C/1 min; 60°C/1 min; 72°C/15 min for 12 cycles), the methylated parental DNA was digested with the restriction enzyme DpnI, leaving only the unmethylated PCR amplified products. The resulting annealed double-stranded nicked products were then transformed into bacteria and the resulting colonies were screened for the desired mutations by sequencing. The subsequent constructs were fully sequenced to verify that the mutations were in fact introduced and that no extraneous mutations were generated.

## Example 8

### ACR III mutant increases ADPase activity

Plasmids containing the mutated and wild type forms of the CD39L4 gene were transfected into COS-7 cells. After two days, protein was purified from the culture medium using a Nickel-NTA resin approach to concentrate the tagged proteins. These proteins were then assayed for ATPase and ADPase activity by measuring the inorganic phosphate released (Wang T-F et al., J. Biol. Chem. 273:24814-24821, 1998). The proteins were incubated in apyrase buffer (15 mM Tris pH 7.4, 135 mM NaCl, 2mM EGTA and 10 mM glucose) for 1 hour at 37 °C with or without 2 mM CaCl<sub>2</sub> or 2 mM MgCl<sub>2</sub>. Phosphatase reactions were initiated by the addition of ADP or ATP to a final concentration of 1 mM. The reaction of inorganic phosphorus with ammonium molybdate in the presence of sulfuric acid, produces an unreduced



phosphomolybdate complex. The absorbance of this complex at 340 nm is directly proportional to the inorganic phosphorus concentration (Daly J. A., and Ertlingshausen G. Clin. Chem. 18:263 (1972) (Sigma Diagnostics)).

As seen in Figure 7, mutations in ACR I and II eliminate activity, whereas the mutations in ACR III increase activity six-fold over wild type. This increased activity therefore offers a greater therapeutic potential, as less protein could be administered to offer the same pharmacological effect. The replacement of three amino acids in the III region (amino acids 167 to 181 in CD39-L4) and the resulting increase in ADPase activity predicts that replacement of additional amino acids within this region by amino acids from the equivalent region of CD39 may also enhance the activity of the protein over wild type CD39L4. The increase in ADPase activity over wild type may also be due to the replacement of only one or two of the three amino acids; this can be confirmed by replacing one or two amino acids at a time.

The polynucleotide and amino acid sequences of a CD39-L4 variant termed ACRIII and having the amino acid substitutions D168-T, S170-Q and L175-F compared to wild type CD39-L4 (SEQ ID NO: 5) are set forth in SEQ ID NOS: 6 and 7, respectively, and in Figure 6.

## Example 9

### ACR III mutant and wild type forms are specific for ADP and not ATP

Both the CD39L4 wild type and the CD39L4 variant with mutations in the ACRIII region hydrolyze ADP. However, when ATP was tested as a substrate, neither the CD39L4 nor the CD39L4 variant catalyzed hydrolysis. In contrast, CD39 as a membrane bound molecule (Marcus et al., The Journal of Clinical Investigation, 99: 1351-1360) or as a genetically engineered soluble form (Gayle et al., The Journal of Clinical Investigation, 101:1851-1858, 1998) is able to hydrolyze both ATP and ADP substrates efficiently. The specificity that both CD39L4 wild type and the CD39L4 variant with mutations in the ACRIII region have for ADP is an advantageous feature that makes these CD39L4-type

molecules better antiplatelet therapeutic candidates than CD39, as ADP is the agonist that causes platelet aggregation. Therapeutics that have both ADPase and ATPase activities potentially could create adverse side effects by interfering with levels of ATP in the circulation.

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### Example 10

#### Organization of the human CD39-L4 gene

A human CITB BAC genomic library (Research Genetics) was screened with gene specific primers [246-I6 (nt 5522-5543), 5'-CTTCCTTCACTGGGAATTCAGG-3' (SEQ ID NO: 18) and 246-K4 (nt 4922-4945), 5'-CTGTTTACCGAGATGGTTGGAAGC-3' (SEQ ID NO: 19)] using a PCR based assay.

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Briefly, gene specific primers were used to screen pools of BAC DNAs. BAC pools that produced a amplified DNA fragment of the predicted size were pursued until an individual BAC was identified. BAC63-I18 was isolated and sequenced with gene specific primers for the CD39-L4 cDNA, as well as intron specific primers. The CD39-L4 coding sequence was found to be distributed over 10 exons spanning 9.3 kb of genomic DNA as set out in SEQ ID NO: 8.

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### Example 11

#### CD39-L4 is stimulated by divalent cations

The high degree of conservation in the apyrase conserved regions of CD39-L4 suggests similar function to other apyrases. To test this hypothesis, COS-7 cells were transfected with the CD39-L4-6His construct as described above. The medium from transfected cells was incubated with Nickel-NTA resin (Qiagen) in order to capture the 6His tagged protein, the resin was washed with assay buffer (buffer A, 15 mM Tris pH 7.5, 134 mM NaCl and 5 mM glucose) and the protein still tethered to the resin in a suspension was assayed for ADPase activity. Nucleotidase activity was

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determined by measuring the amount of inorganic phosphate released from nucleotide substrates using the technique of Day and Ertingshausen, Clin. Chem. **18**:263-265 (1972). In this reaction the complex of inorganic phosphorus with phosphor reagent (ammonium molybdate in the presence of sulfuric acid) produces an unreduced phosphomolybdate compound. The absorbance of this complex at 340 nm is directly proportional in the inorganic phosphorus concentration. The protein still tethered to the resin as a 30% suspension in buffer A was assayed by the addition of the nucleotide to a final concentration of 1 mM and incubated at 37°C for 30 minutes. The reaction was stopped by adding 100 volumes of phosphor reagent. The amount of phosphate released from the reaction was quantified using a calcium/phosphorus combined standard (Sigma). The amount of CD39-L4 protein used in the assays was estimated by comparing the intensity of the CD39-L4 band in Western blots with that of a series of standards of known quantity. CD39-L4 protein from transfected cells displayed a 2.3 fold increase in activity over the cells transfected with the vector alone. When  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  were added, the activity increased 3.6 fold and 6 fold, respectively.

### Example 12

#### Characterization of CD39-L4 activity

CD39-L4 protein was assayed for ADPase activity in the presence of different kinds of inhibitors of ADPases. Control ecto-apyrase activity was determined with protein tethered to the Nickel-NTA resin. Both assays were performed as described above except the protein was in buffer A containing 2 mM  $\text{CaCl}_2$  and 2 mM  $\text{MgCl}_2$ . As shown by Table 1 below, inhibitors of phosphatases ( $\text{F}^-$ ) and adenylate kinase (Ap5A) did not inhibit activity. The inhibitors of vacuolar ATPases (NEM), mitochondrial ATPases ( $\text{N}_3$ ) and  $\text{Na}^+$ ,  $\text{K}^+$ , ATPase (ouabain) did not significantly inhibit the  $\text{Ca}^{2+}$  and  $\text{Mg}^{2+}$  stimulated activity. However, metal chelators (EDTA and EGTA) significantly inhibited activity. These results show that the overwhelming

majority of the activity in the assays originates from a protein bound to the resin with characteristics of an E-type apyrase.

**Table 1**  
**Inhibition of CD39-L4 activity**

INHIBITORS	% OF CONTROL
Control	100 ± 7
Ouabain (1 mM)	96 ± 6
NEM (10 mM)	106 ± 5
N3 <sup>-</sup> (1 mM)	100 ± 12
F <sup>-</sup> (10 mM)	113 ± 5
Ap5A (10 μM)	121 ± 9
EGTA (2 mM)	35 ± 3
EDTA (2 mM)	52 ± 3

As shown in Table 2 below, the nucleotide specificity of CD39-L4 was also assayed as described above. The CD39-L4 activity was determined with protein tethered to the Ni-NTA resin. The protein was in buffer A containing 1 mM EGTA, as well as 2 mM CaCl<sub>2</sub> and MgCl<sub>2</sub>. The assay was started by adding the nucleotides to a final concentration of 1 mM. The values below are expressed relative to ADP. The relative activity of the nucleotide triphosphates varies almost seven-fold with ATP being the poorest substrate. No phosphate release was detected with AMP and ADP was hydrolyzed at a rate approximately twenty-fold higher than ATP. The other nucleotide diphosphates (GDP and UDP) were also very efficiently hydrolyzed by CD39-L4. These results indicate that CD39-L4 defines a new class of E-type apyrase in humans with a specificity for NDPs as enzymatic substrates.

**Table 2**  
**Substrate specificity of CD39-L4**

NUCLEOTIDE	% OF CONTROL
ADP	100 ± 15
ATP	5 ± 1
AMP	0
CTP	26 ± 2
GTP	34 ± 1
UTP	12 ± 4
CDP	268 ± 11
GDP	334 ± 38
UDP	408 ± 14

### Example 13

#### Glycosylation is not essential for CD39-L4 activity

Posttranslational modifications such as N-linked glycosylation are common in secreted and membrane-bound mammalian proteins. These modifications may be important for correct protein folding or enzymatic activity and are not easily reproduced when the proteins are expressed in other organisms such as bacteria. In order to test whether CD39-L4 is glycosylated, COS-7 cells, transfected as described in Example 43, were treated with tunicamycin (Sigma), which blocks the formation of N-glycosidic linkages.

COS-7 cells were grown to 75% confluency and transfected with the CD39-L4-6His construct. After 24 hours, a fraction of the COS-7 cells were treated with Tunicamycin at a concentration of 5 µg/ml. The media was replaced again after 24 hours with fresh tunicamycin and harvested after 48 hours. The CD39-L4-6His protein was concentrated by treating the media with Nickel-NTA agarose (Qiagen). The resin was washed with assay buffer

and the protein still tethered to the resin in a suspension was assayed for a shift in electrophoretic mobility as well as its ADPase activity.

Western blot analysis using an antibody against the 6-His epitope revealed that the glycosylated CD39-L4 protein isolated from the control cells had an approximate size of 51 kDa. However, tunicamycin treated cells had a molecular weight of approximately 46 kDa indicating that the protein was deglycosylated.

ADPase activity of the tunicamycin treated cells was assayed as described in Example 13 above. The deglycosylated CD39-L4 protein had ADPase activity comparable to an equal amount of the glycosylated protein isolated from control cells. This demonstrates that glycosylation of the protein is not important for ADPase activity.

The present invention is not to be limited in scope by the exemplified embodiments which are intended as illustrations of single aspects of the invention, and compositions and methods which are functionally equivalent are within the scope of the invention. Indeed, numerous modifications and variations in the practice of the invention are expected to occur to those skilled in the art upon consideration of the present preferred embodiments. Consequently, the only limitations which should be placed upon the scope of the invention are those which appear in the appended claims.

All references cited within the body of the instant specification are hereby incorporated by reference in their entirety.